

## DESCRIPTION

### ACTIVATION OF LYMPHOCYTE POPULATIONS EXPRESSING NKG2D USING ANTI-NKG2D ANTIBODIES AND LIGAND DERIVATIVES

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#### BACKGROUND OF THE INVENTION

The government owns rights in the present invention pursuant to grant numbers RO1  
AI30581 and POI CA18221 from the National Institutes of Health.

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##### **1. Field of the Invention**

The present invention relates generally to the field of immunology. More particularly, it  
describes stimulation of immune functions through cell surface molecules known as NKG2D,  
which may be targeted to treat cancer, viral diseases and other conditions.

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##### **2. Description of Related Art**

Intracellular antigens, such as viral proteins, are recognized by CD8  $\alpha\beta$  T-cells after they  
are processed to short peptides and presented by polymorphic major histocompatibility complex  
(MHC) class I molecules (Germain & Margulies, 1993). T-cells become activated by  
engagement of their clonotypic T-cell antigen receptor (TCR)-CD3 complexes by specific MHC  
class I-peptide molecules and of the costimulatory CD28 receptor by its CD80-CD86 ligands,  
which are expressed on professional antigen-presenting cells (Davis *et al.*, 1998; Lenschow *et al.*, 1996). Proficient occupation of both receptors results in T-cell proliferation and interleukin  
(IL)-2 production whereas triggering of the TCR-CD3 complex alone favors T-cell anergy or  
apoptosis (Hara *et al.*, 1985; Thompson *et al.*, 1989; Gimmi *et al.*, 1991; Linsley *et al.*, 1991;  
Harding *et al.*, 1992; Gribben *et al.*, 1995; Chambers & Allison, 1999).

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In addition to these central receptor-ligand interactions, diverse adhesion or signaling  
molecules modulate T-cell activation. The latter may include inhibitory or stimulatory receptors  
that were first identified on natural killer (NK) cells, but are also expressed on T-cells. Among  
these are isoforms of the killer cell immunoglobulin (Ig)-like receptors (KIR), which interact  
with MHC class I HLA-A, -B, or -C, and the lectin-like CD94-NKG2A or CD94-NKG2C  
receptor pairs that bind HLA-E (Ravetch & Lanier, 2000; Lee *et al.*, 1998). The inhibitory

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receptors have cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIM) that function by recruitment of tyrosine phosphatases (Long, 1999). Activating isoforms of KIR, which lack ITIM, and the CD94-NKG2C receptor associate with an adaptor protein, DAP12, which signals similar to the CD3 $\zeta$  chain, by activation of tyrosine kinases after phosphorylation of its tyrosine-based activation motif (ITAM) (Lanier *et al.*, 1998). When NK cells engage target cells, the aggregate effects of signals from these and other receptors become integrated to favor inhibition or activation of effector functions (Lanier, 2000). With T-cells, there is evidence that ligand engagement of inhibitory receptors can increase TCR-dependent activation thresholds (Phillips *et al.*, 1995; Carena *et al.*, 1997; Ikeda *et al.*, 1997; Bakker *et al.*, 1998; Noppen *et al.*, 1998); however, whether and how signals from activating receptors are functionally integrated is unknown.

A stimulatory receptor of particular interest is NKG2D, as it is expressed on most NK cells, CD8  $\alpha\beta$  T-cells and  $\gamma\delta$  T-cells, and thus is the most widely distributed "NK cell receptor" known (Bauer *et al.*, 1999). NKG2D shares no close relationships with other NKG2 family members and is not associated with CD94. It forms homodimers that pair with an adaptor protein, DAP10, which may signal by recruitment of phosphatidylinositol-3 kinase (PI3K) upon phosphorylation of a tyrosine-based motif in its cytoplasmic domain (Wu *et al.*, 1999). Whereas the function of KIR and CD94-NKG2 receptors is to monitor the expression of MHC class I molecules, which is often impaired on virus-infected or tumor cells (Ravetch & Lanier, 2000), NKG2D interacts with ligands that are not constitutively but inducibly expressed.

Among these are human MICA and MICB, which are distant homologs of MHC class I, but have no function in antigen presentation (Bahram *et al.*, 1994; Bahram & Spies, 1996; Groh *et al.*, 1996; Li *et al.*, 1999). These molecules are stress-induced similar to heat-shock protein 70 (hsp70), presumably owing to the presence of putative heat-shock elements in the 5'-flanking regions of the corresponding genes (Groh *et al.*, 1996; Groh *et al.*, 1998). They have a restricted tissue distribution in intestinal epithelium and are frequently expressed in epithelial tumors (Groh *et al.*, 1996; Groh *et al.*, 1999). While it is known that engagement of NKG2D by MIC stimulates NK cell and  $\gamma\delta$  T-cell effector functions, and may positively modulate CD8  $\alpha\beta$  T-cell responses (Bauer *et al.*, 1999; Groh *et al.*, 1998), the ability to exploit this knowledge has not been demonstrated.

## SUMMARY OF THE INVENTION

Therefore, in a first embodiment, there is provided a method for expanding a human T-cell population that expresses a natural or engineered NKG2D comprising contacting said  
5 population with an NKG2D ligand. The NKG2D ligand may be an anti-NKG2D antibody, or an NKG2D-binding fragment thereof. The contacting may be performed *in vivo* or *ex vivo*. The anti-NKG2-D antibody fragment may be Fab, F(ab')<sub>2</sub>, or single-chain antibody.

The cell population may be a CD8<sup>+</sup> population or a CD4<sup>+</sup> population, a T cell population, an NK cell population or a monocyte population. Where a T cell population, it may be an  
10 antigen-specific T cell population, for example, from a subject with a primed anti-tumor responder with a primed anti-viral response. The T cell population also may be from an immunocompromised subject. In a further, embodiment, the T cell population may be induced to secrete lymphokines.

In another embodiment, there is provided a method for inducing lymphokine secretion  
15 from a human cell population that expresses a natural or engineered comprising contacting said population with an anti-NKG2-D antibody, or an NKG2-D-binding fragment thereof. The lymphokine may be INF- $\gamma$ , TNF- $\alpha$ , GM-CSF, IL-2 or IL-4.

In still another embodiment, there is provided a method for enhancing an antigen-specific T cell response in a subject comprising (a) obtaining a population of antigen-specific T cells, (b)  
20 contacting said population of antigen-specific T cells with an anti-NKG2-D antibody, or an NKG2-D-binding fragment thereof, and (c) administering said population to said subject.

In still yet another embodiment, there is provided a method for treating cancer comprising (a) obtaining a population of antigen-specific T cells from a subject having cancer, (b) contacting said population of antigen-specific T cells with an anti-NKG2-D antibody, or an  
25 NKG2-D-binding fragment thereof, and (c) administering said population to said subject. The cancer may be an epithelial tumor, for example, a carcinoma such as a carcinoma of the breast, lung, colon, kidney, prostate, or ovary. The cancer also may be a melanoma.

In a further embodiment, this is provided a method for treating a viral infection comprising (a) obtaining a population of antigen-specific T cells from a subject having a viral  
30 infection, (b) contacting said population of antigen-specific T cells with an anti-NKG2-D antibody, or an NKG2-D-binding fragment thereof, and (c) administering said population to said subject.

In still a further embodiment, there is provided a method of stimulating the immune system of an immunocompromised subject comprising (a) obtaining a population of antigen-specific T cells from said subject, (b) contacting said population of antigen-specific T cells with an anti-NKG2-D antibody, or an NKG2-D-binding fragment thereof, and (c) administering said population to said subject.

In yet a further embodiment, there is provided a method of stimulating an effector function a lymphocyte comprising (a) obtaining a population of lymphocytes, and (b) contacting said population of lymphocytes with an anti-NKG2-D antibody, or an NKG2-D-binding fragment thereof.

In an additional embodiment, there is provided a method of stimulating a memory function of a lymphocyte comprising (a) obtaining a population of lymphocytes, and (b) contacting said population of lymphocytes with an anti-NKG2-D antibody, or an NKG2-D-binding fragment thereof.

#### **BRIEF DESCRIPTION OF THE DRAWINGS**

**FIGS. 1A & 1B - Induction of MIC expression on CMV-infected fibroblasts and endothelial cells.** FIG. 1A. With primary human skin fibroblast cultures infected with CMV AD169, staining with mAb 6D4 and flow cytometry showed substantial increases of MIC expression (filled profiles) between 24 (upper panel) and 72 h (bottom panel) after infection while MHC class I (shaded profiles) detected with mAb W6/32 decreased. Similar results were obtained with a number of different anti-MIC mAbs. Open profiles are Ig-isotype control stainings. FIG. 1B. Two-color immunofluorescence stainings of umbilical vein endothelial cells infected at low multiplicity with CMV VHL/e showed two distinct cell populations with inversely correlated surface levels of MIC and MHC class I.

**FIGS. 2A & 2B - Association of induced MIC expression with productive CMV infection in cultured endothelial cells and lung disease.** FIG. 2A. Two-color immunostainings of endothelial cell monolayers partially infected with CMV VHL/e for CMV IE-1 (mAb NEA-9221, visualized by green fluorescence with Streptavidin CY conjugate) and MIC (mAb 6D4, visualized by red fluorescence with Streptavidin Alexa 594 conjugate). Nuclei were stained with diamino-phenylindole. See Methods for technical details. FIG. 2B. Cryostat sections of CMV interstitial pneumonia specimens stained for MIC (large micrograph; brown diamino benzidine

peroxidase substrate staining) or for MIC and CMV delayed early DNA-binding protein p52 (small insert micrograph; additional Fast Red peroxidase substrate staining). Due to technical limitations, better contrast could not be achieved in the two-color tissue stainings, which serve as a complement to the image shown in FIG. 2A. No stainings were observed with sections of control lung specimens.

**FIGS. 3A-F - Augmentation of anti-CMV cytolytic T-cell responses by MICA-NKG2D.** FIGS. 3A & 3B. Primary skin fibroblast cultures typed for HLA-A1 or -A2 expressing unaltered versus increased and decreased amounts of MIC and MHC class I 12 and 72 h after infection with CMV AD169, respectively, were tested as targets for HLA-matched pp65-specific CD28<sup>+</sup> CD8  $\alpha\beta$  T-cell clones in chromium release assays. Fluorescence profiles in histograms are labeled according to the time points of MIC or MHC class I antibody staining. Open profiles are Ig-isotype control stainings. FIGS. 3C & 3F. At 12 h post-infection, the cytolytic activities of the T-cell clones 8E8-403 (HLA-A1) and 19D1-66 (HLA-A2) could be inhibited by anti-MHC class I (mAb W6/32) but not by anti-MIC (mAb 6D4) or anti-NKG2D (mAb 1D11). At 72 h post-infection, mAbs against MIC or NKG2D had inhibitory effects. Similar data were obtained with additional two HLA-A1- and five HLA-A2-restricted T-cell clones (see Methods). FIGS. 3D & 3E. No lysis was scored with HLA-mismatched combinations of T-cells and virus-infected targets. Ranges of standard deviations (SD) are indicated above bars in percent.

**FIG. 4 - Antigen dose-dependent augmentation of cytolytic T-cell function by NKG2D.** Cytotoxic responses of pp65-specific T-cells against C1R-A2-MICA double transfectants pulsed with the HLA-A2-restricted NLVPMVATV peptide were substantially stronger than those against identically treated C1R-A2 transfectants within a range of suboptimal peptide concentrations. These increases were diminished by mAb against MICA or NKG2D. The results obtained with the 4H6-254 T-cell clone were representative of five T-cell clones tested. All assays were done in triplicate with deviations that were not greater than about 3%.

**FIGS. 5A-D - Stimulation of T-cell cytokine secretion by NKG2D.** C1R-A2-MICA cells pulsed with the specific pp65 peptide stimulated secretion of much larger amounts of (FIG. 5A) IFN- $\gamma$ , (FIG. 5B) TNF- $\alpha$ , (FIG. 5C) IL-2, and (FIG. 5D) IL-4 by the HLA-A2-restricted pp65-specific T-cell clone 2E9-269 than C1R-A2 cells pulsed with the same peptide concentrations. Note that in the absence of MICA on the stimulator cells no IL-2 was detected in T-cell supernatants. The results shown were similar to those obtained with four other T-cell clones (see Methods) and for GM-CSF and IL-4 (data not shown). Each bar represents the

cytokine ELISA read-out from three pooled wells of T-cell supernatants. All of these assays, including parallel experiments with anti-NKG2D, anti-MIC or isotype control antibody (data not shown), were performed three times with comparable results. The total number of data points (bars) was 3240 (12 bars/graph x 5 T-cell clones x 6 cytokines x 3 antibodies x 3 experiments).

5        **FIGS. 6A-C - Stimulation by NKG2D of IL-2 production in peripheral blood CMV-specific CD28<sup>-</sup> CD8  $\alpha\beta$  T-cells.** FIG. 6A. Among CD8  $\alpha\beta$  T-cells isolated by negative selection from peripheral blood, pp65-specific T-cells were identified by fluorescence staining with HLA-A2 tetramers refolded with pp65 peptide and flow cytometry. The gated CD28<sup>-</sup> population of these T-cells included a proportion of cells that stained positively for intracellular IL-2 after  
10   short-term coculture with peptide-pulsed C1R-A2-MICA cells (FIG. 6C) but not after identical coculture with peptide-pulsed C1R-A2 cells lacking MIC (FIG. 6B). See Methods for further technical details.

**FIGS. 7A-C - Costimulation by NKG2D of TCR-CD3 complex-dependent IL-2 production and proliferation of CD28<sup>-</sup> CD8  $\alpha\beta$  T-cells.** FIG. 7A. Triggering of the T-cell  
15   clone 4H6-254, which was representative of five T-cell clones tested, with a range of concentrations of plate-bound anti-CD3 mAb resulted in minimal or modest T-cell proliferation measured by [<sup>3</sup>H]thymidine incorporation. However, T-cell proliferation was strongly amplified in the additional presence of solid-phase anti-NKG2D (mAb 1D11) but not of Ig-isotype control antibody. FIG. 7B. Combined triggering with anti-CD3 and anti-NKG2D potently induced T-cell  
20   IL-2 secretion. Data shown are representative of five T-cell clones tested. FIG. 7C. Anti-NKG2D in combination with anti-CD3 superinduced proliferation of freshly isolated peripheral blood CD28<sup>-</sup> CD8  $\alpha\beta$  T-cells. Experiments in FIG. 7A & 7B were done in triplicate with no more than about 3% deviation.

## 25        DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

### I.        **MIC BINDING TO NKG2D**

      The present invention stems, in part, from the inventors' earlier discoveries of the existence and function of MICA and MICB and of their role as ligands for NKG2D. Herein, the  
30   significance of MIC immunobiology is again demonstrated by showing that MIC expression is induced by human cytomegalovirus (CMV) infection, and further, that engagement of the NKG2D receptor by MIC strongly augments anti-CMV CD8  $\alpha\beta$  T-cell responses despite the

viral interference with antigen presentation. This probably represents an important factor in the immunological control of this virus, which establishes lifelong persistence marked by alternating periods of latency and reactivation in infected hosts, and can likely be extrapolated to at least some other viral and microbial infections.

5        Notably, a recent report has suggested that a CMV glycoprotein, UL16, which interacts with MIC and a set of cell surface proteins termed ULBP, may interfere with NKG2D function. Posnett *et al.* (1999). If substantiated, this would lend further support to the hypothesis that MIC-NKG2D may effectively enable the immune system to combat this virus. Moreover, because MIC expression is associated with diverse epithelial tumors including lung, breast,  
10    colon, ovary, prostate and renal cell carcinomas (Groh *et al.*, 1999), these results indicate that their interaction with NKG2D may also stimulate responses by CD8  $\alpha\beta$  T-cells specific for tumor antigens. Together, these results support the model that the MIC-NKG2D system, with its ability to activate NK cells and T-cells, may function as an emergency defense against infectious agents and hazardous conditions that cause cellular distress.

15        The NKG2D-mediated augmentation of effector T-cell responses, such as cytotoxicity and secretion of IFN- $\gamma$  and TNF- $\alpha$ , presumably involves ligand adhesion as indicated by the strong binding of soluble MICA to cell surface NKG2D (Bauer *et al.* 1999). More significantly, however, NKG2D potently stimulates TCR-CD3 complex-dependent T-cell proliferation and IL-2 production. Thus, NKG2D functions as a costimulatory receptor although its mechanism of  
20    signaling *via* DAP10 may not have been completely resolved. These results highlight the significance of MIC expression throughout the gastrointestinal epithelium (Groh *et al.*, 1996), implying that this site may have costimulatory capacity.

      Among peripheral effector CD8  $\alpha\beta$  T-cells, about 20-60% are negative for CD28, depending on age and factors such as chronic infections (Posnett *et al.*, 1999). These T-cells have  
25    been found hyporesponsive to stimulation by anti-CD3 even in the presence of exogenously added IL-2 (Azuma *et al.*, 1993). The current results show that ligand engagement of NKG2D can reverse this anergic state and rescue autocrine proliferation. This indicates that triggering of NKG2D by suitably engineered derivatives of antibodies or ligands can be applied to effectively expand specific effector CD8  $\alpha\beta$  T-cells *in vitro* and to boost primed T-cell responses by local  
30    targeting or systemic administration *in vivo*. The inventors have previously reported that MICS function as antigens for a subset of  $\gamma\delta$  T-cells (V $\delta$ 1  $\gamma\delta$  T-cells) that predominates in epithelial sites (Groh *et al.*, 1998; Groh *et al.*, 1999). Thus, the current evidence suggests that, in the

activation of these T-cells, MIC may provide signal 1 (TCR-dependent) as well as signal 2 (NKG2D-dependent).

Because of the broad distribution among lymphocyte subsets and functional potency of NKG2D, it appears imperative that the expression of its ligands must be tightly controlled to limit T-cell proliferation and avert autoimmune reactions. By the same token, the substantial expression of MIC on large proportions of gastrointestinal epithelium suggests that NKG2D may be regulated as well to minimize the risk of widespread inflammation. In addition to MICA and MICB, NKG2D interacts with other ligands that have disparate sequences although they share common MHC class I-like  $\alpha 1\alpha 2$  domains. These include the putative human ULBP proteins and their possible murine counterparts - the retinoic acid early inducible RAE-1 family of ligands (Chalupny *et al.*, 2000; Cerwenka *et al.*, 2000; Diefenbach *et al.*, 2000). As of yet, little is known about the immunologically relevant expression of these molecules, except that they may be present on some tumor cells (Diefenbach *et al.*, 2000).

## II. NKG2D

Major histocompatibility complex class I molecules are ligands for inhibitory or activating natural killer (NK) cell receptors that are expressed on NK cells and T cells. These include three isoforms of the immunoglobulin (Ig)-like killer cell receptors that interact with HLA-A, -B or -C, and CD94 paired with NKG2A or NKG2C, which bind HLA-E. Engagement of these receptors modulates NK cell responses and TCR-dependent T-cell activation.

In 1999, Bauer *et al.* identified NKG2D as a receptor for stress-induced MICA. NKG2D had previously been proposed to have an activating function because of the lack of a tyrosine-based inhibitory motif in its cytoplasmic tail. In addition, it was known that NKG2D's partner, DAP10, interacts with the p85 subunit of PI3-kinase. The study by Bauer *et al.* used soluble MICA in binding assays, representational difference analysis (RDA) and protein immunoprecipitation with specific monoclonal antibodies to show that NKG2D is a receptor for MICA. Its apparently molecular mass of 42 kD matched independent data obtained with polyclonal antibodies.

NKG2D lacks a tyrosine-based inhibitory motif in its cytoplasmic tail and may function as an activating receptor; signaling may be enabled by DAP10, which has an SH2 domain-binding site for the p85 subunit of phosphoinositide 3-kinase. An activating function is supported by the inhibition of  $\gamma\delta$  T-cell recognition of MICA mediated by monoclonal antibody against  $\gamma\delta$  T-



cell receptor. However, these responses can also be inhibited by monoclonal antibodies against  $\gamma\delta$  T-cell receptors, implying that their activation also requires T-cell receptor engagement.

To examine whether NKG2D can function in the absence of T-cell receptor signaling, Bauer *et al.* (1999) used NK cell effectors. These showed the expected cytotoxicity against Daudi cells, which lack  $\beta_2$ -microglobulin ( $\beta_2m$ ) and thus MHC class I, whereas Daudi- $\beta_2m$  transfectants were protected by the restored expression of MHC class I; inhibition of KNL was mediated by HLA-E, the ligand for CD94-NKG2A. However, coexpression of MICA sensitized Daudi- $\beta_2m$  cells to lysis, which could be inhibited by anti-MICA and anti-NKG2D antibody. MICA did not diminish surface expression of class I. Hence, masking of HLA-E on Daudi- $\beta_2m$ -MICA cells increased cytolysis to a level above that recorded with Daudi cells. Ligation of NKG2D on NK cells with monoclonal antibodies induced redirected lysis of Fc receptor (FcR)-bearing cells, similar to responses with anti-CD16. Thus, in agreement with its broad distribution on most  $\gamma\delta$  T-cells, CD8<sup>+</sup>  $\alpha\beta$  T cells and NK cells, NKG2D has an activating function triggered by engagement of MICA (or presumably of MICB) over a diverse range of effector cells.

DNA sequences for NKG2D can be found in WO 92/17198, incorporated herein by reference. NKG2D genes, and their corresponding cDNA can be inserted into an appropriate cloning vehicle for manipulation thereof. In addition, sequence variants of the polypeptide may be utilized. These may, for instance, be minor sequence variants of the polypeptide that arise due to natural variation within the population or they may be homologues found in other species. They also may be sequences that do not occur naturally but that are sufficiently similar that they function similarly and/or elicit an immune response that cross-reacts with natural forms of the polypeptide. Sequence variants can be prepared by standard methods of site-directed mutagenesis such as those described below in the following section.

#### A. Variants of NKG2D

Amino acid sequence variants of NKG2D can be substitutional, insertional or deletion variants. Substitutional variants typically contain the exchange of one amino acid for another at one or more sites within the protein, and may be designed to modulate one or more properties of the polypeptide such as stability against proteolytic cleavage. Substitutions preferably are conservative, that is, one amino acid is replaced with one of similar shape and charge. Conservative substitutions are well known in the art and include, for example, the changes of: alanine to serine; arginine to

lysine; asparagine to glutamine or histidine; aspartate to glutamate; cysteine to serine; glutamine to asparagine; glutamate to aspartate; glycine to proline; histidine to asparagine or glutamine; isoleucine to leucine or valine; leucine to valine or isoleucine; lysine to arginine; methionine to leucine or isoleucine; phenylalanine to tyrosine, leucine or methionine; serine to threonine; 5 threonine to serine; tryptophan to tyrosine; tyrosine to tryptophan or phenylalanine; and valine to isoleucine or leucine.

Insertional variants include fusion proteins such as those used to allow rapid purification of the polypeptide and also can include hybrid proteins containing sequences from other proteins and polypeptides which are homologues of the polypeptide. For example, an insertional variant could 10 include portions of the amino acid sequence of the polypeptide from one species, together with portions of the homologous polypeptide from another species. Other insertional variants can include those in which additional amino acids are introduced within the coding sequence of the polypeptide. These typically are smaller insertions than the fusion proteins described above and are introduced, for example, into a protease cleavage site.

For example, certain amino acids may be substituted for other amino acids in a protein structure without appreciable loss of interactive binding capacity with structures such as, for example, antigen-binding regions of antibodies or binding sites on substrate molecules. Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid substitutions can be made in a protein sequence, and its underlying 15 DNA coding sequence, and nevertheless obtain a protein with like properties. It is thus contemplated by the inventors that various changes may be made in the DNA sequences of genes without appreciable loss of their biological utility or activity. Table 1 shows the codons that encode particular amino acids. 20

In making such changes, the hydropathic index of amino acids may be considered. The 25 importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in the art (Kyte & Doolittle, 1982).

**TABLE 1**

Amino Acids			Codons					
Alanine	Ala	A	GCA	GCC	GCG	GCU		
Cysteine	Cys	C	UGC	UGU				
Aspartic acid	Asp	D	GAC	GAU				
Glutamic acid	Glu	E	GAA	GAG				
Phenylalanine	Phe	F	UUC	UUU				
Glycine	Gly	G	GGA	GGC	GGG	GGU		
Histidine	His	H	CAC	CAU				
Isoleucine	Ile	I	AUA	AUC	AUU			
Lysine	Lys	K	AAA	AAG				
Leucine	Leu	L	UUA	UUG	CUA	CUC	CUG	CUU
Methionine	Met	M	AUG					
Asparagine	Asn	N	AAC	AAU				
Proline	Pro	P	CCA	CCC	CCG	CCU		
Glutamine	Gln	Q	CAA	CAG				
Arginine	Arg	R	AGA	AGG	CGA	CGC	CGG	CGU
Serine	Ser	S	AGC	AGU	UCA	UCC	UCG	UCU
Threonine	Thr	T	ACA	ACC	ACG	ACU		
Valine	Val	V	GUA	GUC	GUG	GUU		
Tryptophan	Trp	W	UGG					
Tyrosine	Tyr	Y	UAC	UAU				

5           It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like.

10          Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics (Kyte & Doolittle, 1982), these are: Isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3);

**TABLE 1**

Amino Acids			Codons					
Alanine	Ala	A	GCA	GCC	GCG	GCU		
Cysteine	Cys	C	UGC	UGU				
Aspartic acid	Asp	D	GAC	GAU				
Glutamic acid	Glu	E	GAA	GAG				
Phenylalanine	Phe	F	UUC	UUU				
Glycine	Gly	G	GGA	GGC	GGG	GGU		
Histidine	His	H	CAC	CAU				
Isoleucine	Ile	I	AUA	AUC	AUU			
Lysine	Lys	K	AAA	AAG				
Leucine	Leu	L	UUA	UUG	CUA	CUC	CUG	CUU
Methionine	Met	M	AUG					
Asparagine	Asn	N	AAC	AAU				
Proline	Pro	P	CCA	CCC	CCG	CCU		
Glutamine	Gln	Q	CAA	CAG				
Arginine	Arg	R	AGA	AGG	CGA	CGC	CGG	CGU
Serine	Ser	S	AGC	AGU	UCA	UCC	UCG	UCU
Threonine	Thr	T	ACA	ACC	ACG	ACU		
Valine	Val	V	GUA	GUC	GUG	GUU		
Tryptophan	Trp	W	UGG					
Tyrosine	Tyr	Y	UAC	UAU				

5           It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like.

10           Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics (Kyte & Doolittle, 1982), these are: Isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3);

proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

It is known in the art that certain amino acids may be substituted by other amino acids having a similar hydropathic index or score and still result in a protein with similar biological activity, *i.e.*, still obtain a biological functionally equivalent protein. In making such changes, the substitution of amino acids whose hydropathic indices are within  $\pm 2$  is preferred, those which are within  $\pm 1$  are particularly preferred, and those within  $\pm 0.5$  are even more particularly preferred.

It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. U.S. Patent 4,554,101, incorporated herein by reference, states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein.

As detailed in U.S. Patent 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0  $\pm$  1); glutamate (+3.0  $\pm$  1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5  $\pm$  1); alanine (-0.5); histidine \*-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4).

It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically equivalent and immunologically equivalent protein. In such changes, the substitution of amino acids whose hydrophilicity values are within  $\pm 2$  is preferred, those that are within  $\pm 1$  are particularly preferred, and those within  $\pm 0.5$  are even more particularly preferred.

As outlined above, amino acid substitutions are generally based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions that take various of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

## **B. Fusion Proteins**

Within one embodiment of the invention, specific fusion proteins of NKG2D are contemplated. By fusing the external domain of NKG2D with a distinct DAP10 interacting domain or with cytoplasmic domains derived from other signaling molecules, for example CD28,

one may be able to engineer cells that respond to NKG2D ligands and potentially create a system with enhanced signaling capabilities. Alternatively, one may link transmembrane or cytoplasmic domains from NKG2D with distinct extracellular ligand binding domains. This permits “designer” cells to be created that respond to alternative signaling molecules.

5

### III. LIGANDS FOR NKG2D

#### A. MICA and MICB

MICA and MICB are natural ligands for NKG2D. Although MICA and MICB are encoded by genes in the MHC, they share only about 27% amino acid sequence identity with conventional MHC class I chains in their extracellular  $\alpha 1\alpha 2\alpha 3$  domains. MICA/B themselves are closely related, sharing 84% identical amino acids (Bahram *et al.*, 1994; Bahram & Spies, 1996). Unlike MHC class I, the highly glycosylated MICA/B surface proteins are not associated  $\beta_2$ -microglobulin and peptides and lack the main CD8 binding site (Groh *et al.*, 1996). The crystal structure MICA revealed a dramatically altered MHC class I fold in which the membrane-distal  $\alpha 1\alpha 2$  superdomain is flexibly linked to the Ig-like  $\alpha 3$  domain, such that all of its surfaces including the underside of the  $\beta$ -pleated sheet are accessible for potential molecular interactions. The  $\alpha 1\alpha 2$  helices on top of the  $\beta$ -strand platform are highly distorted and do not form a potential ligand-binding groove (Li *et al.*, 1999). These distortions are similar to those in the mouse nonclassical MHC class I T22 molecule, which has been shown to interact with a small subset of  $\gamma\delta$  T-cells from murine spleen. Sequences directly related to MICA/B are conserved in the genomes of most, if not all, mammalian species with the possible exception of rodents, and are expressed in all of a number of diverse non-human primates that have been investigated (Bahram *et al.*, 1994).

Unlike MHC class I molecules, which are ubiquitously expressed, the distribution of MICA/B proteins in normal tissues is restricted to intestinal epithelium. Notably, the 5'-end of flanking regions of both genes include putative heat-shock elements similar to those in hsp70 genes (Groh *et al.*, 1996). Heat shock treatment of epithelial cell lines grown under conditions of minimal cell proliferation results in potent increases of MICA/B mRNA and surface protein expression (Groh *et al.*, 1998). Possibly associated with this apparent stress-inducible regulation, MICA/B have been found variably expressed in many, but not all, epithelial tumors including lung, breast, kidney, ovary, prostate and colon carcinomas (Groh *et al.* 1999).

## **B. Other Natural Ligands**

Several other binding ligands for NKG2D include the human ULBP proteins and their possible murine counterparts - the retinoic acid early inducible RAE-1 family of ligands (Chalupny *et al.*, 2000; Cerwenka *et al.*, 2000; Diefenbach *et al.*, 2000. These molecules, or fragments or derivatives thereof, may be used to stimulate NKG2D in a fashion analogous to MICA/B.

## **C. Antibodies**

The present inventors have successfully produced monoclonal antibodies that bind specifically to NKG2D. In particular, the antibodies 1D11 (ATCC Deposit No. PTA-3056, deposited Feb. 15, 2001) and 5C6 (ATCC Deposit No. PTA-3055, deposited Feb. 15, 2001) are suitable for all of the disclosed methods. Polyclonal antibodies and other monoclonal antibodies may be produced that may be utilized according to the present invention. For therapeutic purposes, antibodies may be humanized and/or otherwise manipulated to optimize efficacy.

## **D. Mimetics**

In addition to the biological functional equivalents discussed above, the present inventors also contemplate that structurally similar compounds may be formulated to mimic the key portions of peptide or polypeptides of the present invention. Such compounds, which may be termed peptidomimetics, may be used in the same manner as the peptides of the invention and, hence, also are functional equivalents.

Certain mimetics that mimic elements of protein secondary and tertiary structure are described in Johnson *et al.* (1993). The underlying rationale behind the use of peptide mimetics is that the peptide backbone of proteins exists chiefly to orient amino acid side chains in such a way as to facilitate molecular interactions, such as those of antibody and/or antigen. A peptide mimetic is thus designed to permit molecular interactions similar to the natural molecule.

Some successful applications of the peptide mimetic concept have focused on mimetics of  $\beta$ -turns within proteins, which are known to be highly antigenic. Likely  $\beta$ -turn structure within a polypeptide can be predicted by computer-based algorithms, as discussed herein. Once the component amino acids of the turn are determined, mimetics can be constructed to achieve a similar spatial orientation of the essential elements of the amino acid side chains.

Other approaches have focused on the use of small, multidisulfide-containing proteins as attractive structural templates for producing biologically active conformations that mimic the binding sites of large proteins (Vita *et al.*, 1998). A structural motif that appears to be evolutionarily conserved in certain toxins is small (30-40 amino acids), stable, and high permissive for mutation. This motif is composed of a  $\beta$  sheet and an alpha helix bridged in the interior core by three disulfides.

Beta II turns have been mimicked successfully using cyclic L-pentapeptides and those with D-amino acids (Weisshoff *et al.*, 1999). Also, Johannesson *et al.* (1999) report on bicyclic tripeptides with reverse turn inducing properties. Methods for generating specific structures have been disclosed in the art. For example, alpha-helix mimetics are disclosed in U.S. Patents 5,446,128; 5,710,245; 5,840,833; and 5,859,184. These structures render the peptide or protein more thermally stable, also increase resistance to proteolytic degradation. Six, seven, eleven, twelve, thirteen and fourteen membered ring structures are disclosed.

Methods for generating conformationally restricted beta turns and beta bulges are described, for example, in U.S. Patents 5,440,013; 5,618,914; and 5,670,155. Beta-turns permit changed side substituents without having changes in corresponding backbone conformation, and have appropriate termini for incorporation into peptides by standard synthesis procedures. Other types of mimetic turns include reverse and gamma turns. Reverse turn mimetics are disclosed in U.S. Patents 5,475,085 and 5,929,237, and gamma turn mimetics are described in U.S. Patents 5,672,681 and 5,674,976.

#### **E. Purification of Protein Ligands**

In most embodiments, purification of protein ligands for use according to the present invention will be required. Generally, "purified" will refer to a protein or peptide composition that has been subjected to fractionation to remove various other components, and which composition substantially retains its expressed biological activity. Where the term "substantially purified" is used, this designation will refer to a composition in which the protein or peptide forms the major component of the composition, such as constituting about 50% or more of the proteins in the composition.

Various methods for quantifying the degree of purification of the protein or peptide will be known to those of skill in the art in light of the present disclosure. These include, for example, determining the specific activity of an active fraction, or assessing the amount of polypeptides



within a fraction by SDS/PAGE analysis. A preferred method for assessing the purity of a fraction is to calculate the specific activity of the fraction, to compare it to the specific activity of the initial extract, and to thus calculate the degree of purity, herein assessed by a "-fold purification number." The actual units used to represent the amount of activity will, of course, be dependent upon the particular assay technique chosen to follow the purification and whether or not the expressed protein or peptide exhibits a detectable activity.

Various techniques suitable for use in protein purification will be well known to those of skill in the art. These include, for example, precipitation with ammonium sulphate, PEG, antibodies and the like or by heat denaturation, followed by centrifugation; chromatography steps such as ion exchange, gel filtration, reverse phase, hydroxylapatite and affinity chromatography; isoelectric focusing; gel electrophoresis; and combinations of such and other techniques. As is generally known in the art, it is believed that the order of conducting the various purification steps may be changed, or that certain steps may be omitted, and still result in a suitable method for the preparation of a substantially purified protein or peptide.

There is no general requirement that the protein or peptide always be provided in their most purified state. Indeed, it is contemplated that less substantially purified products will have utility in certain embodiments. Partial purification may be accomplished by using fewer purification steps in combination, or by utilizing different forms of the same general purification scheme. For example, it is appreciated that a cation-exchange column chromatography performed utilizing an HPLC apparatus will generally result in a greater -fold purification than the same technique utilizing a low pressure chromatography system. Methods exhibiting a lower degree of relative purification may have advantages in total recovery of protein product, or in maintaining the activity of an expressed protein.

It is known that the migration of a polypeptide can vary, sometimes significantly, with different conditions of SDS/PAGE (Capaldi *et al.*, 1977). It will therefore be appreciated that under differing electrophoresis conditions, the apparent molecular weights of purified or partially purified expression products may vary.

High Performance Liquid Chromatography (HPLC) is characterized by a very rapid separation with extraordinary resolution of peaks. This is achieved by the use of very fine particles and high pressure to maintain an adequate flow rate. Separation can be accomplished in a matter of minutes, or at most an hour. Moreover, only a very small volume of the sample is needed because the particles are so small and close-packed that the void volume is a very small

fraction of the bed volume. Also, the concentration of the sample need not be very great because the bands are so narrow that there is very little dilution of the sample.

5        Gel chromatography, or molecular sieve chromatography, is a special type of partition chromatography that is based on molecular size. The theory behind gel chromatography is that the column, which is prepared with tiny particles of an inert substance that contain small pores, separates larger molecules from smaller molecules as they pass through or around the pores, depending on their size. As long as the material of which the particles are made does not adsorb the molecules, the sole factor determining rate of flow is the size. Hence, molecules are eluted from the column in decreasing size, so long as the shape is relatively constant. Gel  
10       chromatography is unsurpassed for separating molecules of different size because separation is independent of all other factors such as pH, ionic strength, temperature, *etc.* There also is virtually no adsorption, less zone spreading and the elution volume is related in a simple matter to molecular weight.

15       Affinity Chromatography is a chromatographic procedure that relies on the specific affinity between a substance to be isolated and a molecule that it can specifically bind to. This is a receptor-ligand type interaction. The column material is synthesized by covalently coupling one of the binding partners to an insoluble matrix. The column material is then able to specifically adsorb the substance from the solution. Elution occurs by changing the conditions to those in which binding will not occur (alter pH, ionic strength, temperature, *etc.*).

20       A particular type of affinity chromatography useful in the purification of carbohydrate containing compounds is lectin affinity chromatography. Lectins are a class of substances that bind to a variety of polysaccharides and glycoproteins. Lectins are usually coupled to agarose by cyanogen bromide. Concanavalin A coupled to Sepharose was the first material of this sort to be used and has been widely used in the isolation of polysaccharides and glycoproteins other lectins  
25       that have been include lentil lectin, wheat germ agglutinin which has been useful in the purification of N-acetyl glucosaminyl residues and *Helix pomatia* lectin. Lectins themselves are purified using affinity chromatography with carbohydrate ligands. Lactose has been used to purify lectins from castor bean and peanuts; maltose has been useful in extracting lectins from lentils and jack bean; N-acetyl-D galactosamine is used for purifying lectins from soybean; N-  
30       acetyl glucosaminyl binds to lectins from wheat germ; D-galactosamine has been used in obtaining lectins from clams and L-fucose will bind to lectins from lotus.

The matrix should be a substance that itself does not adsorb molecules to any significant extent and that has a broad range of chemical, physical and thermal stability. The ligand should be coupled in such a way as to not affect its binding properties. The ligand should also provide relatively tight binding. And it should be possible to elute the substance without destroying the sample or the ligand. One of the most common forms of affinity chromatography is immunoaffinity chromatography. The generation of antibodies that would be suitable for use in accord with the present invention is discussed below.

#### IV. ANTIBODY PRODUCTION

##### A. Generation of Monoclonal Antibodies

In another aspect, the present invention contemplates an antibody that is immunoreactive with NKG2D extracellular domains. An antibody can be a polyclonal or a monoclonal antibody. In a preferred embodiment, an antibody is a monoclonal antibody. Means for preparing and characterizing antibodies are well known in the art (see, *e.g.*, Howell and Lane, 1988).

Briefly, a polyclonal antibody is prepared by immunizing an animal with an immunogen comprising a polypeptide of the present invention and collecting antisera from that immunized animal. A wide range of animal species can be used for the production of antisera. Typically an animal used for production of anti-antisera is a non-human animal including rabbits, mice, rats, hamsters, pigs or horses. Because of the relatively large blood volume of rabbits, a rabbit is a preferred choice for production of polyclonal antibodies.

Antibodies, both polyclonal and monoclonal, specific for isoforms of antigen may be prepared using conventional immunization techniques, as will be generally known to those of skill in the art. A composition containing antigenic epitopes of the compounds of the present invention can be used to immunize one or more experimental animals, such as a rabbit or mouse, which will then proceed to produce specific antibodies against the compounds of the present invention. Polyclonal antisera may be obtained, after allowing time for antibody generation, simply by bleeding the animal and preparing serum samples from the whole blood.

Additionally, it is proposed that monoclonal antibodies specific to the particular NKG2D alleles may be utilized in other useful applications. For example, their use in immunoabsorbent protocols may be useful in purifying native or recombinant NKG2D isoforms or variants thereof.

In general, both poly- and monoclonal antibodies against NKG2D-related antigens may be used in a variety of embodiments. For example, they may be employed in antibody cloning

protocols to obtain cDNAs or genes encoding NKG2D or fragments thereof. Means for preparing and characterizing antibodies are well known in the art (See, e.g., Harlow and Lane, 1988; incorporated herein by reference). More specific examples of monoclonal antibody preparation are give in the examples below.

5 As is well known in the art, a given composition may vary in its immunogenicity. It is often necessary therefore to boost the host immune system, as may be achieved by coupling a peptide or polypeptide immunogen to a carrier. Exemplary and preferred carriers are keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA). Other albumins such as ovalbumin, mouse serum albumin or rabbit serum albumin can also be used as carriers. Means  
10 for conjugating a polypeptide to a carrier protein are well known in the art and include glutaraldehyde, *m*-maleimidobencoyl-N-hydroxysuccinimide ester, carbodiimide and bis-biazotized benzidine.

As is also well known in the art, the immunogenicity of a particular immunogen composition can be enhanced by the use of non-specific stimulators of the immune response,  
15 known as adjuvants. Exemplary and preferred adjuvants include complete Freund's adjuvant (a non-specific stimulator of the immune response containing killed *Mycobacterium tuberculosis*), incomplete Freund's adjuvants and aluminum hydroxide adjuvant.

The amount of immunogen composition used in the production of polyclonal antibodies varies upon the nature of the immunogen as well as the animal used for immunization. A variety  
20 of routes can be used to administer the immunogen (subcutaneous, intramuscular, intradermal, intravenous and intraperitoneal). The production of polyclonal antibodies may be monitored by sampling blood of the immunized animal at various points following immunization. A second, booster, injection may also be given. The process of boosting and titering is repeated until a suitable titer is achieved. When a desired level of immunogenicity is obtained, the immunized  
25 animal can be bled and the serum isolated and stored, and/or the animal can be used to generate mAbs.

MAbs may be readily prepared through use of well-known techniques, such as those exemplified in U.S. Patent 4,196,265, incorporated herein by reference. Typically, this technique involves immunizing a suitable animal with a selected immunogen composition, e.g., a purified  
30 or partially purified NKG2D protein, polypeptide or peptide or cell expressing high levels of NKG2D. The immunizing composition is administered in a manner effective to stimulate antibody producing cells. Rodents such as mice and rats are preferred animals, however, the use

of rabbit, sheep frog cells is also possible. The use of rats may provide certain advantages (Goding, 1986), but mice are preferred, with the BALB/c mouse being most preferred as this is most routinely used and generally gives a higher percentage of stable fusions.

Following immunization, somatic cells with the potential for producing antibodies, specifically B-lymphocytes (B-cells), are selected for use in the mAb generating protocol. These cells may be obtained from biopsied spleens, tonsils or lymph nodes, or from a peripheral blood sample. Spleen cells and peripheral blood cells are preferred, the former because they are a rich source of antibody-producing cells that are in the dividing plasmablast stage, and the latter because peripheral blood is easily accessible. Often, a panel of animals will have been immunized and the spleen of animal with the highest antibody titer will be removed and the spleen lymphocytes obtained by homogenizing the spleen with a syringe. Typically, a spleen from an immunized mouse contains approximately  $5 \times 10^7$  to  $2 \times 10^8$  lymphocytes.

The antibody-producing B lymphocytes from the immunized animal are then fused with cells of an immortal myeloma cell, generally one of the same species as the animal that was immunized. Myeloma cell lines suited for use in hybridoma-producing fusion procedures preferably are non-antibody-producing, have high fusion efficiency, and enzyme deficiencies that render them incapable of growing in certain selective media which support the growth of only the desired fused cells (hybridomas).

Any one of a number of myeloma cells may be used, as are known to those of skill in the art (Goding, 1986; Campbell, 1984). For example, where the immunized animal is a mouse, one may use P3-X63/Ag8, P3-X63-Ag8.653, NS1/1.Ag 4 1, Sp210-Ag14, FO, NSO/U, MPC-11, MPC11-X45-GTG 1.7 and S194/5XX0 Bul; for rats, one may use R210.RCY3, Y3-Ag 1.2.3, IR983F and 4B210; and U-266, GM1500-GRG2, LICR-LON-HMy2 and UC729-6 are all useful in connection with cell fusions.

Methods for generating hybrids of antibody-producing spleen or lymph node cells and myeloma cells usually comprise mixing somatic cells with myeloma cells in a 2:1 ratio, though the ratio may vary from about 20:1 to about 1:1, respectively, in the presence of an agent or agents (chemical or electrical) that promote the fusion of cell membranes. Fusion methods using Sendai virus have been described (Kohler and Milstein, 1975; 1976), and those using polyethylene glycol (PEG), such as 37% (v/v) PEG, by Gefter *et al.*, (1977). The use of electrically induced fusion methods is also appropriate (Goding, 1986).

Fusion procedures usually produce viable hybrids at low frequencies, around  $1 \times 10^{-6}$  to  $1 \times 10^{-8}$ . However, this does not pose a problem, as the viable, fused hybrids are differentiated from the parental, unfused cells (particularly the unfused myeloma cells that would normally continue to divide indefinitely) by culturing in a selective medium. The selective medium is

5 generally one that contains an agent that blocks the *de novo* synthesis of nucleotides in the tissue culture media. Exemplary and preferred agents are aminopterin, methotrexate, and azaserine. Aminopterin and methotrexate block *de novo* synthesis of both purines and pyrimidines, whereas azaserine blocks only purine synthesis. Where aminopterin or methotrexate is used, the media is supplemented with hypoxanthine and thymidine as a source of nucleotides (HAT medium).

10 Where azaserine is used, the media is supplemented with hypoxanthine.

The preferred selection medium is HAT. Only cells capable of operating nucleotide salvage pathways are able to survive in HAT medium. The myeloma cells are defective in key enzymes of the salvage pathway, *e.g.*, hypoxanthine phosphoribosyl transferase (HPRT), and they cannot survive. The B-cells can operate this pathway, but they have a limited life span in

15 culture and generally die within about two weeks. Therefore, the only cells that can survive in the selective media are those hybrids formed from myeloma and B-cells.

This culturing provides a population of hybridomas from which specific hybridomas are selected. Typically, selection of hybridomas is performed by culturing the cells by single-clone dilution in microtiter plates, followed by testing the individual clonal supernatants (after about

20 two to three weeks) for the desired reactivity. The assay should be sensitive, simple and rapid, such as radioimmunoassays, enzyme immunoassays, cytotoxicity assays, plaque assays, dot immunobinding assays, and the like.

The selected hybridomas would then be serially diluted and cloned into individual antibody-producing cell lines, which clones can then be propagated indefinitely to provide

25 mAbs. The cell lines may be exploited for mAb production in two basic ways. A sample of the hybridoma can be injected (often into the peritoneal cavity) into a histocompatible animal of the type that was used to provide the somatic and myeloma cells for the original fusion. The injected animal develops tumors secreting the specific monoclonal antibody produced by the fused cell hybrid. The body fluids of the animal, such as serum or ascites fluid, can then be tapped to

30 provide mAbs in high concentration. The individual cell lines could also be cultured *in vitro*, where the mAbs are naturally secreted into the culture medium from which they can be readily obtained in high concentrations. mAbs produced by either means may be further purified, if

desired, using filtration, centrifugation and various chromatographic methods such as HPLC or affinity chromatography.

## V. CELLS

### A. NKG2D Expressing Cells

The present invention, in one embodiment, will employ cells that naturally express NKG2D. Such cells include most  $\gamma\delta$  T-cells, CD8<sup>+</sup>  $\alpha\beta$  T cells and NK cells. In other contexts, cells may be engineered to express NKG2D, or a suitable derivative thereof. General attributes of cells suitable for such engineering include any antigen-specific or regulatory T-cells (CD8 or CD4  $\alpha\beta$  T-cells) that are expanded *in vitro*, transduced for enhanced or *de novo* expression of NKG2D or a suitable fusion protein and infused into patients for treatment of tumors or viral or other microbial diseases.

### B. Expression Constructs

The term "expression vector" or "expression construct" is used to refer to a carrier nucleic acid molecule into which a nucleic acid sequence can be inserted for introduction into a cell where it can be replicated. A nucleic acid sequence can be "exogenous," which means that it is foreign to the cell into which the vector is being introduced or that the sequence is homologous to a sequence in the cell but in a position within the host cell nucleic acid in which the sequence is ordinarily not found. Vectors include plasmids, cosmids, viruses (bacteriophage, animal viruses, and plant viruses), and artificial chromosomes (*e.g.*, YACs). One of skill in the art would be well equipped to construct a vector through standard recombinant techniques (see, for example, Maniatis *et al.*, 1988 and Ausubel *et al.*, 1994, both incorporated herein by reference).

These terms refer to any type of genetic construct comprising a nucleic acid coding for a RNA capable of being transcribed. In some cases, RNA molecules are then translated into a protein, polypeptide, or peptide. In other cases, these sequences are not translated, for example, in the production of antisense molecules or ribozymes. Expression vectors can contain a variety of "control sequences," which refer to nucleic acid sequences necessary for the transcription and possibly translation of an operably linked coding sequence in a particular host cell. In addition to control sequences that govern transcription and translation, vectors and expression vectors may contain nucleic acid sequences that serve other functions as well and are described *infra*.

### i) Promoters and Enhancers

A "promoter" is a control sequence that is a region of a nucleic acid sequence at which initiation and rate of transcription are controlled. It may contain genetic elements at which regulatory proteins and molecules may bind, such as RNA polymerase and other transcription factors, to initiate the specific transcription a nucleic acid sequence. The phrases "operatively positioned," "operatively linked," "under control," and "under transcriptional control" mean that a promoter is in a correct functional location and/or orientation in relation to a nucleic acid sequence to control transcriptional initiation and/or expression of that sequence.

A promoter generally comprises a sequence that functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as, for example, the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation. Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have been shown to contain functional elements downstream of the start site as well. To bring a coding sequence "under the control of" a promoter, one positions the 5' end of the transcription initiation site of the transcriptional reading frame "downstream" of (*i.e.*, 3' of) the chosen promoter. The "upstream" promoter stimulates transcription of the DNA and promotes expression of the encoded RNA.

The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either cooperatively or independently to activate transcription. A promoter may or may not be used in conjunction with an "enhancer," which refers to a cis-acting regulatory sequence involved in the transcriptional activation of a nucleic acid sequence.

A promoter may be one naturally associated with a nucleic acid sequence, as may be obtained by isolating the 5' non-coding sequences located upstream of the coding segment and/or exon. Such a promoter can be referred to as "endogenous." Similarly, an enhancer may be one naturally associated with a nucleic acid sequence, located either downstream or upstream of that sequence. Alternatively, certain advantages will be gained by positioning the coding nucleic acid segment under the control of a recombinant or heterologous promoter, which refers to a promoter



that is not normally associated with a nucleic acid sequence in its natural environment. A recombinant or heterologous enhancer refers also to an enhancer not normally associated with a nucleic acid sequence in its natural environment. Such promoters or enhancers may include promoters or enhancers of other genes, and promoters or enhancers isolated from any other virus, or prokaryotic or eukaryotic cell, and promoters or enhancers not "naturally occurring," *i.e.*, containing different elements of different transcriptional regulatory regions, and/or mutations that alter expression. For example, promoters that are most commonly used in recombinant DNA construction include the  $\beta$ -lactamase (penicillinase), lactose and tryptophan (trp) promoter systems. In addition to producing nucleic acid sequences of promoters and enhancers synthetically, sequences may be produced using recombinant cloning and/or nucleic acid amplification technology, including PCR<sup>TM</sup>, in connection with the compositions disclosed herein (see U.S. Patents 4,683,202 and 5,928,906, each incorporated herein by reference). Furthermore, it is contemplated the control sequences that direct transcription and/or expression of sequences within non-nuclear organelles such as mitochondria, chloroplasts, and the like, can be employed as well.

Naturally, it will be important to employ a promoter and/or enhancer that effectively directs the expression of the DNA segment in the organelle, cell type, tissue, organ, or organism chosen for expression. Those of skill in the art of molecular biology generally know the use of promoters, enhancers, and cell type combinations for protein expression, (see, for example Sambrook *et al.* 1989, incorporated herein by reference). The promoters employed may be constitutive, tissue-specific, inducible, and/or useful under the appropriate conditions to direct high level expression of the introduced DNA segment, such as is advantageous in the large-scale production of recombinant proteins and/or peptides. The promoter may be heterologous or endogenous.

Additionally any promoter/enhancer combination (as per, for example, the Eukaryotic Promoter Data Base EPDB, <http://www.epd.isb-sib.ch/>) could also be used to drive expression. Use of a T3, T7 or SP6 cytoplasmic expression system is another possible embodiment. Eukaryotic cells can support cytoplasmic transcription from certain bacterial promoters if the appropriate bacterial polymerase is provided, either as part of the delivery complex or as an additional genetic expression construct.

Table 2 lists non-limiting examples of elements/promoters that may be employed, in the context of the present invention, to regulate the expression of a RNA. Table 3 provides non-

limiting examples of inducible elements, which are regions of a nucleic acid sequence that can be activated in response to a specific stimulus.

Table 2	
Promoter and/or Enhancer	
Promoter/Enhancer	References
Immunoglobulin Heavy Chain	Banerji <i>et al.</i> , 1983; Gilles <i>et al.</i> , 1983; Grosschedl <i>et al.</i> , 1985; Atchinson <i>et al.</i> , 1986, 1987; Imler <i>et al.</i> , 1987; Weinberger <i>et al.</i> , 1984; Kiledjian <i>et al.</i> , 1988; Porton <i>et al.</i> ; 1990
Immunoglobulin Light Chain	Queen <i>et al.</i> , 1983; Picard <i>et al.</i> , 1984
T-Cell Receptor	Luria <i>et al.</i> , 1987; Winoto <i>et al.</i> , 1989; Redondo <i>et al.</i> ; 1990
HLA DQ a and/or DQ $\beta$	Sullivan <i>et al.</i> , 1987
$\beta$ -Interferon	Goodbourn <i>et al.</i> , 1986; Fujita <i>et al.</i> , 1987; Goodbourn <i>et al.</i> , 1988
Interleukin-2	Greene <i>et al.</i> , 1989
Interleukin-2 Receptor	Greene <i>et al.</i> , 1989; Lin <i>et al.</i> , 1990
MHC Class II 5	Koch <i>et al.</i> , 1989
MHC Class II HLA-Dra	Sherman <i>et al.</i> , 1989
$\beta$ -Actin	Kawamoto <i>et al.</i> , 1988; Ng <i>et al.</i> ; 1989
Muscle Creatine Kinase (MCK)	Jaynes <i>et al.</i> , 1988; Horlick <i>et al.</i> , 1989; Johnson <i>et al.</i> , 1989
Prealbumin (Transferrin)	Costa <i>et al.</i> , 1988
Elastase I	Ornitz <i>et al.</i> , 1987
Metallothionein (MTII)	Karin <i>et al.</i> , 1987; Culotta <i>et al.</i> , 1989
Collagenase	Pinkert <i>et al.</i> , 1987; Angel <i>et al.</i> , 1987
Albumin	Pinkert <i>et al.</i> , 1987; Tronche <i>et al.</i> , 1989, 1990
$\alpha$ -Fetoprotein	Godbout <i>et al.</i> , 1988; Campere <i>et al.</i> , 1989
$\gamma$ -Globin	Bodine <i>et al.</i> , 1987; Perez-Stable <i>et al.</i> , 1990
$\beta$ -Globin	Trudel <i>et al.</i> , 1987
c-fos	Cohen <i>et al.</i> , 1987
c-HA-ras	Triesman, 1986; Deschamps <i>et al.</i> , 1985
Insulin	Edlund <i>et al.</i> , 1985

Table 2	
Promoter and/or Enhancer	
Promoter/Enhancer	References
Neural Cell Adhesion Molecule (NCAM)	Hirsch <i>et al.</i> , 1990
$\alpha_1$ -Antitrypsin	Latimer <i>et al.</i> , 1990
H2B (TH2B) Histone	Hwang <i>et al.</i> , 1990
Mouse and/or Type I Collagen	Ripe <i>et al.</i> , 1989
Glucose-Regulated Proteins (GRP94 and GRP78)	Chang <i>et al.</i> , 1989
Rat Growth Hormone	Larsen <i>et al.</i> , 1986
Human Serum Amyloid A (SAA)	Edbrooke <i>et al.</i> , 1989
Troponin I (TN I)	Yutzey <i>et al.</i> , 1989
Platelet-Derived Growth Factor (PDGF)	Pech <i>et al.</i> , 1989
Duchenne Muscular Dystrophy	Klamut <i>et al.</i> , 1990
SV40	Banerji <i>et al.</i> , 1981; Moreau <i>et al.</i> , 1981; Sleight <i>et al.</i> , 1985; Firak <i>et al.</i> , 1986; Herr <i>et al.</i> , 1986; Imbra <i>et al.</i> , 1986; Kadesch <i>et al.</i> , 1986; Wang <i>et al.</i> , 1986; Ondek <i>et al.</i> , 1987; Kuhl <i>et al.</i> , 1987; Schaffner <i>et al.</i> , 1988
Polyoma	Swartzendruber <i>et al.</i> , 1975; Vasseur <i>et al.</i> , 1980; Katinka <i>et al.</i> , 1980, 1981; Tyndell <i>et al.</i> , 1981; Dandolo <i>et al.</i> , 1983; de Villiers <i>et al.</i> , 1984; Hen <i>et al.</i> , 1986; Satake <i>et al.</i> , 1988; Campbell and/or Villarreal, 1988
Retroviruses	Kriegler <i>et al.</i> , 1982, 1983; Levinson <i>et al.</i> , 1982; Kriegler <i>et al.</i> , 1983, 1984a, b, 1988; Bosze <i>et al.</i> , 1986; Miksicsek <i>et al.</i> , 1986; Celander <i>et al.</i> , 1987; Thiesen <i>et al.</i> , 1988; Celander <i>et al.</i> , 1988; Choi <i>et al.</i> , 1988; Reisman <i>et al.</i> , 1989
Papilloma Virus	Campo <i>et al.</i> , 1983; Lusky <i>et al.</i> , 1983; Spandidos and/or Wilkie, 1983; Spalholz <i>et al.</i> , 1985; Lusky <i>et al.</i> , 1986; Cripe <i>et al.</i> , 1987; Gloss <i>et al.</i> , 1987; Hirochika <i>et al.</i> , 1987; Stephens <i>et al.</i> , 1987
Hepatitis B Virus	Bulla <i>et al.</i> , 1986; Jameel <i>et al.</i> , 1986; Shaul <i>et al.</i> , 1987; Spandau <i>et al.</i> , 1988; Vannice <i>et al.</i> , 1988
Human Immunodeficiency Virus	Muesing <i>et al.</i> , 1987; Hauber <i>et al.</i> , 1988; Jakobovits <i>et al.</i> , 1988; Feng <i>et al.</i> , 1988; Takebe <i>et al.</i> , 1988; Rosen <i>et al.</i> , 1988; Berkhout <i>et al.</i> , 1989; Laspia <i>et al.</i> , 1989; Sharp <i>et al.</i> , 1989; Braddock <i>et al.</i> , 1989

Table 2	
Promoter and/or Enhancer	
Promoter/Enhancer	References
Cytomegalovirus (CMV)	Weber <i>et al.</i> , 1984; Boshart <i>et al.</i> , 1985; Foecking <i>et al.</i> , 1986
Gibbon Ape Leukemia Virus	Holbrook <i>et al.</i> , 1987; Quinn <i>et al.</i> , 1989

Table 3		
Inducible Elements		
Element	Inducer	References
MT II	Phorbol Ester (TFA) Heavy metals	Palmiter <i>et al.</i> , 1982; Haslinger <i>et al.</i> , 1985; Searle <i>et al.</i> , 1985; Stuart <i>et al.</i> , 1985; Imagawa <i>et al.</i> , 1987, Karin <i>et al.</i> , 1987; Angel <i>et al.</i> , 1987b; McNeall <i>et al.</i> , 1989
MMTV (mouse mammary tumor virus)	Glucocorticoids	Huang <i>et al.</i> , 1981; Lee <i>et al.</i> , 1981; Majors <i>et al.</i> , 1983; Chandler <i>et al.</i> , 1983; Lee <i>et al.</i> , 1984; Ponta <i>et al.</i> , 1985; Sakai <i>et al.</i> , 1988
$\beta$ -Interferon	Poly(rI)x Poly(rc)	Tavernier <i>et al.</i> , 1983
Adenovirus 5 <u>E2</u>	E1A	Imperiale <i>et al.</i> , 1984
Collagenase	Phorbol Ester (TPA)	Angel <i>et al.</i> , 1987a
Stromelysin	Phorbol Ester (TPA)	Angel <i>et al.</i> , 1987b
SV40	Phorbol Ester (TPA)	Angel <i>et al.</i> , 1987b
Murine MX Gene	Interferon, Newcastle Disease Virus	Hug <i>et al.</i> , 1988
GRP78 Gene	A23187	Resendez <i>et al.</i> , 1988
$\alpha$ -2-Macroglobulin	IL-6	Kunz <i>et al.</i> , 1989
Vimentin	Serum	Rittling <i>et al.</i> , 1989
MHC Class I Gene H-2kb	Interferon	Blonar <i>et al.</i> , 1989
HSP70	E1A, SV40 Large T	Taylor <i>et al.</i> , 1989, 1990a, 1990b

Table 3		
Inducible Elements		
Element	Inducer	References
	Antigen	
Proliferin	Phorbol Ester-TPA	Mordacq <i>et al.</i> , 1989
Tumor Necrosis Factor $\alpha$	PMA	Hensel <i>et al.</i> , 1989
Thyroid Stimulating Hormone $\alpha$ Gene	Thyroid Hormone	Chatterjee <i>et al.</i> , 1989

The identity of tissue-specific promoters or elements, as well as assays to characterize their activity, is well known to those of skill in the art. Nonlimiting examples of such regions include the human LIMK2 gene (Nomoto *et al.* 1999), the somatostatin receptor 2 gene (Kraus *et al.*, 1998), murine epididymal retinoic acid-binding gene (Lareyre *et al.*, 1999), human CD4 (Zhao-Emonet *et al.*, 1998), mouse alpha2 (XI) collagen (Tsumaki, *et al.*, 1998), D1A dopamine receptor gene (Lee, *et al.*, 1997), insulin-like growth factor II (Wu *et al.*, 1997), and human platelet endothelial cell adhesion molecule-1 (Almendo *et al.*, 1996).

#### ii) Initiation Signals and Internal Ribosome Binding Sites

A specific initiation signal also may be required for efficient translation of coding sequences. These signals include the ATG initiation codon or adjacent sequences. Exogenous translational control signals, including the ATG initiation codon, may need to be provided. One of ordinary skill in the art would readily be capable of determining this and providing the necessary signals. It is well known that the initiation codon must be "in-frame" with the reading frame of the desired coding sequence to ensure translation of the entire insert. The exogenous translational control signals and initiation codons can be either natural or synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements.

In certain embodiments of the invention, the use of internal ribosome entry sites (IRES) elements are used to create multigene, or polycistronic, messages. IRES elements are able to bypass the ribosome scanning model of 5' methylated Cap dependent translation and begin translation at internal sites (Pelletier and Sonenberg, 1988). IRES elements from two members of the picornavirus family (polio and encephalomyocarditis) have been described (Pelletier and

Sonenberg, 1988), as well as an IRES from a mammalian message (Macejak and Sarnow, 1991). IRES elements can be linked to heterologous open reading frames. Multiple open reading frames can be transcribed together, each separated by an IRES, creating polycistronic messages. By virtue of the IRES element, each open reading frame is accessible to ribosomes for efficient translation. Multiple genes can be efficiently expressed using a single promoter/enhancer to transcribe a single message (see U.S. Patents 5,925,565 and 5,935,819, each herein incorporated by reference).

### iii) Multiple Cloning Sites

Vectors can include a multiple cloning site (MCS), which is a nucleic acid region that contains multiple restriction enzyme sites, any of which can be used in conjunction with standard recombinant technology to digest the vector (see, for example, Carbonelli *et al.* 1999; Levenson *et al.* 1998; and Cocea 1997, incorporated herein by reference). "Restriction enzyme digestion" refers to catalytic cleavage of a nucleic acid molecule with an enzyme that functions only at specific locations in a nucleic acid molecule. Many of these restriction enzymes are commercially available. Use of such enzymes is widely understood by those of skill in the art. Frequently, a vector is linearized or fragmented using a restriction enzyme that cuts within the MCS to enable exogenous sequences to be ligated to the vector. "Ligation" refers to the process of forming phosphodiester bonds between two nucleic acid fragments, which may or may not be contiguous with each other. Techniques involving restriction enzymes and ligation reactions are well known to those of skill in the art of recombinant technology.

### iv) Splicing Sites

Most transcribed eukaryotic RNA molecules will undergo RNA splicing to remove introns from the primary transcripts. Vectors containing genomic eukaryotic sequences may require donor and/or acceptor splicing sites to ensure proper processing of the transcript for protein expression (see, for example, Chandler *et al.*, 1997, herein incorporated by reference).

### v) Termination Signals

The vectors or constructs of the present invention will generally comprise at least one termination signal. A "termination signal" or "terminator" is comprised of the DNA sequences involved in specific termination of an RNA transcript by an RNA polymerase. Thus, in certain

embodiments a termination signal that ends the production of an RNA transcript is contemplated. A terminator may be necessary *in vivo* to achieve desirable message levels.

In eukaryotic systems, the terminator region may also comprise specific DNA sequences that permit site-specific cleavage of the new transcript so as to expose a polyadenylation site.

5 This signals a specialized endogenous polymerase to add a stretch of about 200 A residues (polyA) to the 3' end of the transcript. RNA molecules modified with this polyA tail appear to more stable and are translated more efficiently. Thus, in other embodiments involving eukaryotes, it is preferred that that terminator comprises a signal for the cleavage of the RNA, and it is more preferred that the terminator signal promotes polyadenylation of the message. The  
10 terminator and/or polyadenylation site elements can serve to enhance message levels and to minimize read through from the cassette into other sequences.

Terminators contemplated for use in the invention include any known terminator of transcription described herein or known to one of ordinary skill in the art, including but not limited to, for example, the termination sequences of genes, such as for example the bovine  
15 growth hormone terminator or viral termination sequences, such as for example the SV40 terminator. In certain embodiments, the termination signal may be a lack of transcribable or translatable sequence, such as due to a sequence truncation.

#### vi) Polyadenylation Signals

20 In expression, particularly eukaryotic expression, one will typically include a polyadenylation signal to effect proper polyadenylation of the transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed. Preferred embodiments include the SV40 polyadenylation signal or the bovine growth hormone polyadenylation signal, convenient and  
25 known to function well in various target cells. Polyadenylation may increase the stability of the transcript or may facilitate cytoplasmic transport.

#### vii) Origins of Replication

In order to propagate a vector in a host cell, it may contain one or more origins of  
30 replication sites (often termed "ori"), which is a specific nucleic acid sequence at which replication is initiated. Alternatively an autonomously replicating sequence (ARS) can be employed if the host cell is yeast.

#### viii) Selectable and Screenable Markers

In certain embodiments of the invention, cells containing a nucleic acid construct of the present invention may be identified *in vitro* or *in vivo* by including a marker in the expression  
5 vector. Such markers would confer an identifiable change to the cell permitting easy identification of cells containing the expression vector. Generally, a selectable marker is one that confers a property that allows for selection. A positive selectable marker is one in which the presence of the marker allows for its selection, while a negative selectable marker is one in which its presence prevents its selection. An example of a positive selectable marker is a drug  
10 resistance marker.

Usually the inclusion of a drug selection marker aids in the cloning and identification of transformants, for example, genes that confer resistance to neomycin, puromycin, hygromycin, DHFR, GPT, zeocin and histidinol are useful selectable markers. In addition to markers conferring a phenotype that allows for the discrimination of transformants based on the  
15 implementation of conditions, other types of markers including screenable markers such as GFP, whose basis is colorimetric analysis, are also contemplated. Alternatively, screenable enzymes such as herpes simplex virus thymidine kinase (*tk*) or chloramphenicol acetyltransferase (CAT) may be utilized. One of skill in the art would also know how to employ immunologic markers, possibly in conjunction with FACS analysis. The marker used is not believed to be important, so  
20 long as it is capable of being expressed simultaneously with the nucleic acid encoding a gene product. Further examples of selectable and screenable markers are well known to one of skill in the art.

#### ix) Plasmid Vectors

25 In certain embodiments, a plasmid vector is contemplated for use to transform a host cell. In general, plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell are used in connection with these hosts. The vector ordinarily carries a replication site, as well as marking sequences which are capable of providing phenotypic selection in transformed cells. In a non-limiting example, *E. coli* is often transformed  
30 using derivatives of pBR322, a plasmid derived from an *E. coli* species. pBR322 contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. The pBR plasmid, or other microbial plasmid or phage must also contain, or



be modified to contain, for example, promoters which can be used by the microbial organism for expression of its own proteins.

In addition, phage vectors containing replicon and control sequences that are compatible with the host microorganism can be used as transforming vectors in connection with these hosts.

5 For example, the phage lambda GEM<sup>TM</sup>-11 may be utilized in making a recombinant phage vector which can be used to transform host cells, such as, for example, *E. coli* LE392.

Further useful plasmid vectors include pIN vectors (Inouye *et al.*, 1985); and pGEX vectors, for use in generating glutathione S-transferase (GST) soluble fusion proteins for later purification and separation or cleavage. Other suitable fusion proteins are those with  
10  $\beta$ -galactosidase, ubiquitin, and the like.

Bacterial host cells, for example, *E. coli*, comprising the expression vector, are grown in any of a number of suitable media, for example, LB. The expression of the recombinant protein in certain vectors may be induced, as would be understood by those of skill in the art, by contacting a host cell with an agent specific for certain promoters, e.g., by adding IPTG to the  
15 media or by switching incubation to a higher temperature. After culturing the bacteria for a further period, generally of between 2 and 24 h, the cells are collected by centrifugation and washed to remove residual media.

#### x) Viral Vectors

20 The ability of certain viruses to infect cells or enter cells *via* receptor-mediated endocytosis, and to integrate into host cell genome and express viral genes stably and efficiently have made them attractive candidates for the transfer of foreign nucleic acids into cells. Non-limiting examples of virus vectors that may be used to deliver a nucleic acid of the present invention are described below.

25

##### 1. Adenoviral Vectors

A particular method for delivery of the nucleic acid involves the use of an adenovirus expression vector. Although adenovirus vectors are known to have a low capacity for integration into genomic DNA, this feature is counterbalanced by the high efficiency of gene transfer  
30 afforded by these vectors. "Adenovirus expression vector" is meant to include those constructs containing adenovirus sequences sufficient to (a) support packaging of the construct and (b) to ultimately express a tissue or cell-specific construct that has been cloned therein. Knowledge of

the genetic organization of adenovirus, a 36 kb, linear, double-stranded DNA virus, allows substitution of large pieces of adenoviral DNA with foreign sequences up to 7 kb (Grunhaus and Horwitz 1992).

## 2. AAV Vectors

The nucleic acid may be introduced into the cell using adenovirus assisted transfection. Increased transfection efficiencies have been reported in cell systems using adenovirus coupled systems (Kelleher and Vos 1994; Cotten *et al.* 1992; Curiel 1994). Adeno-associated virus (AAV) is an attractive vector system for use according to the present invention as it has a high frequency of integration and it can infect nondividing cells, thus making it useful for delivery of genes into mammalian cells, for example, in tissue culture (Muzyczka 1992) or *in vivo*. AAV has a broad host range for infectivity (Tratschin *et al.* 1984; Laughlin *et al.* 1986; Lebkowski *et al.* 1988; McLaughlin *et al.* 1988). Details concerning the generation and use of rAAV vectors are described in U.S. Patents 5,139,941 and 4,797,368, each incorporated herein by reference.

## 3. Retroviral Vectors

Retroviruses integrate their genes into the host genome have the advantage of transferring a large amount of foreign genetic material, infecting a broad spectrum of species and cell types, and of being packaged in special cell-lines (Miller, 1992).

In order to construct a retroviral vector, a nucleic acid of interest is inserted into the viral genome in the place of certain viral sequences to produce a virus that is replication-defective. In order to produce virions, a packaging cell line containing the gag, pol, and env genes but without the LTR and packaging components is constructed (Mann *et al.*, 1983). When a recombinant plasmid containing a cDNA, together with the retroviral LTR and packaging sequences is introduced into a special cell line (*e.g.*, by calcium phosphate precipitation for example), the packaging sequence allows the RNA transcript of the recombinant plasmid to be packaged into viral particles, which are then secreted into the culture media (Nicolas and Rubenstein, 1988; Temin, 1986; Mann *et al.*, 1983). The media containing the recombinant retroviruses is then collected, optionally concentrated, and used for gene transfer. Retroviral vectors are able to infect a broad variety of cell types. However, integration and stable expression require the division of host cells (Paskind *et al.*, 1975).

Lentiviruses are complex retroviruses, which, in addition to the common retroviral genes *gag*, *pol*, and *env*, contain other genes with regulatory or structural function. Lentiviral vectors are well known in the art (see, for example, Naldini *et al.*, 1996; Zufferey *et al.*, 1997; Blomer *et al.*, 1997; U.S. Patents 6,013,516 and 5,994,136). Some examples of lentivirus include the Human Immunodeficiency Viruses: HIV-1, HIV-2 and the Simian Immunodeficiency Virus: SIV. Lentiviral vectors have been generated by multiply attenuating the HIV virulence genes, for example, the genes *env*, *vif*, *vpr*, *vpu* and *nef* are deleted making the vector biologically safe.

Recombinant lentiviral vectors are capable of infecting non-dividing cells and can be used for both *in vivo* and *ex vivo* gene transfer and expression of nucleic acid sequences. For example, recombinant lentivirus capable of infecting a non-dividing cell wherein a suitable host cell is transfected with two or more vectors carrying the packaging functions, namely *gag*, *pol* and *env*, as well as *rev* and *tat* is described in U.S. Patent 5,994,136, incorporated herein by reference. One may target the recombinant virus by linkage of the envelope protein with an antibody or a particular ligand for targeting to a receptor of a particular cell-type. By inserting a sequence (including a regulatory region) of interest into the viral vector, along with another gene which encodes the ligand for a receptor on a specific target cell, for example, the vector is now target-specific.

#### 4. Other Viral Vectors

Other viral vectors may be employed as delivery constructs in the present invention. Vectors derived from viruses such as vaccinia virus (Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.*, 1988), sindbis virus, cytomegalovirus and herpes simplex virus may be employed. They offer several attractive features for various mammalian cells (Friedmann, 1989; Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.*, 1988; Horwich *et al.*, 1990).

#### 5. Delivery Using Modified Viruses

A nucleic acid to be delivered may be housed within an infective virus that has been engineered to express a specific binding ligand. The virus particle will thus bind specifically to the cognate receptors of the target cell and deliver the contents to the cell. A novel approach designed to allow specific targeting of retrovirus vectors was developed based on the chemical modification of a retrovirus by the chemical addition of lactose residues to the viral envelope.

This modification can permit the specific infection of hepatocytes *via* sialoglycoprotein receptors.

Another approach to targeting of recombinant retroviruses was designed in which biotinylated antibodies against a retroviral envelope protein and against a specific cell receptor were used. The antibodies were coupled *via* the biotin components by using streptavidin (Roux *et al.*, 1989). Using antibodies against major histocompatibility complex class I and class II antigens, they demonstrated the infection of a variety of human cells that bore those surface antigens with an ecotropic virus *in vitro* (Roux *et al.*, 1989).

### C. Methods for Transforming Host Cells

There are a number of ways in which nucleic acids may introduced into cells. Viral methods rely on the use of viral vectors listed above. A variety of non-viral transduction methods, are outlined below.

Suitable methods for nucleic acid delivery for transformation of an organelle, a cell, a tissue or an organism for use with the current invention are believed to include virtually any method by which a nucleic acid (*e.g.*, DNA) can be introduced into an organelle, a cell, a tissue or an organism, as described herein or as would be known to one of ordinary skill in the art. Such methods include, but are not limited to, direct delivery of DNA such as by *ex vivo* transfection (Wilson *et al.*, 1989, Nabel *et al.*, 1989), by injection (U.S. Patents 5,994,624, 5,981,274, 5,945,100, 5,780,448, 5,736,524, 5,702,932, 5,656,610, 5,589,466 and 5,580,859, each incorporated herein by reference), including microinjection (Harlan and Weintraub, 1985; U.S. Patent 5,789,215, incorporated herein by reference); by electroporation (U.S. Patent 5,384,253, incorporated herein by reference; Tur-Kaspa *et al.* 1986; Potter *et al.*, 1984); by calcium phosphate precipitation (Graham and Van Der Eb 1973; Chen and Okayama, 1987; Rippe *et al.*, 1990); by using DEAE-dextran followed by polyethylene glycol (Gopal, 1985); by direct sonic loading (Fechheimer *et al.* 1987); by liposome mediated transfection (Nicolau and Sene, 1982; Fraley *et al.*, 1979; Nicolau *et al.*, 1987; Wong *et al.*, 1980; Kaneda *et al.*, 1989; Kato *et al.*, 1991) and receptor-mediated transfection (Wu and Wu, 1987; Wu and Wu, 1988); by microprojectile bombardment (PCT Application Nos. WO 94/09699 and 95/06128; U.S. Patents 5,610,042; 5,322,783 5,563,055, 5,550,318, 5,538,877 and 5,538,880, and each incorporated herein by reference); by agitation with silicon carbide fibers (Kaeppler *et al.*, 1990; U.S. Patents 5,302,523 and 5,464,765, each incorporated herein by reference); by *Agrobacterium*-mediated

transformation (U.S. Patents 5,591,616 and 5,563,055, each incorporated herein by reference); by PEG-mediated transformation of protoplasts (Omirulleh *et al.* 1993; U.S. Patents 4,684,611 and 4,952,500, each incorporated herein by reference); by desiccation/inhibition-mediated DNA uptake (Potrykus *et al.*, 1985), and any combination of such methods. Through the application of techniques such as these, organelle(s), cell(s), tissue(s) or organism(s) may be stably or transiently transformed.

i) *Ex Vivo* Transformation

Methods for transfecting vascular cells and tissues removed from an organism in an *ex vivo* setting are known to those of skill in the art. For example, canine endothelial cells have been genetically altered by retroviral gene transfer in vitro and transplanted into a canine (Wilson *et al.*, 1989). In another example, yucatan minipig endothelial cells were transfected by retrovirus in vitro and transplanted into an artery using a double-balloon catheter (Nabel *et al.*, 1989). Thus, it is contemplated that cells or tissues may be removed and transfected *ex vivo* using the nucleic acids of the present invention. In particular aspects, the transplanted cells or tissues may be placed into an organism. In preferred facets, a nucleic acid is expressed in the transplanted cells or tissues.

ii) Injection

In certain embodiments, a nucleic acid may be delivered to an organelle, a cell, a tissue or an organism via one or more injections (*i.e.*, a needle injection), such as, for example, subcutaneously, intradermally, intramuscularly, intravenously, intraperitoneally, *etc.* Methods of injection of vaccines are well known to those of ordinary skill in the art (*e.g.*, injection of a composition comprising a saline solution). Further embodiments of the present invention include the introduction of a nucleic acid by direct microinjection. Direct microinjection has been used to introduce nucleic acid constructs into *Xenopus* oocytes (Harland and Weintraub 1985). The amount of DNA used may vary upon the nature of the antigen as well as the organelle, cell, tissue or organism used.

iii) Electroporation

In certain embodiments of the present invention, a nucleic acid is introduced into an organelle, a cell, a tissue or an organism *via* electroporation. Electroporation involves the

exposure of a suspension of cells and DNA to a high-voltage electric discharge. In some variants of this method, certain cell wall-degrading enzymes, such as pectin-degrading enzymes, are employed to render the target recipient cells more susceptible to transformation by electroporation than untreated cells (U.S. Patent 5,384,253, incorporated herein by reference).

5 Alternatively, recipient cells can be made more susceptible to transformation by mechanical wounding.

Transfection of eukaryotic cells using electroporation has been quite successful. Mouse pre-B lymphocytes have been transfected with human kappa-immunoglobulin genes (Potter *et al.* 1984), and rat hepatocytes have been transfected with the chloramphenicol acetyltransferase gene  
10 (Tur-Kaspa *et al.* 1986) in this manner.

#### iv) Calcium Phosphate

In other embodiments of the present invention, a nucleic acid is introduced to the cells using calcium phosphate precipitation. Human KB cells have been transfected with adenovirus 5  
15 DNA (Graham and Van Der Eb 1973) using this technique. Also in this manner, mouse L(A9), mouse C127, CHO, CV-1, BHK, NIH3T3 and HeLa cells were transfected with a neomycin marker gene (Chen and Okayama 1987), and rat hepatocytes were transfected with a variety of marker genes (Rippe *et al.* 1990).

#### 20 v) DEAE-Dextran

In another embodiment, a nucleic acid is delivered into a cell using DEAE-dextran followed by polyethylene glycol. In this manner, reporter plasmids were introduced into mouse myeloma and erythroleukemia cells (Gopal 1985).

#### 25 vi) Sonication Loading

Additional embodiments of the present invention include the introduction of a nucleic acid by direct sonic loading. LTK<sup>-</sup> fibroblasts have been transfected with the thymidine kinase gene by sonication loading (Fechheimer *et al.* 1987).

#### 30 vii) Liposome-Mediated Transfection

In a further embodiment of the invention, a nucleic acid may be entrapped in a lipid complex such as, for example, a liposome. Liposomes are vesicular structures characterized by a

phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh and Bachhawat 1991). Also contemplated is a nucleic acid complexed with Lipofectamine (Gibco BRL) or Superfect (Qiagen).

Liposome-mediated nucleic acid delivery and expression of foreign DNA *in vitro* has been very successful (Nicolau and Sene 1982; Fraley *et al.* 1979; Nicolau *et al.* 1987). The feasibility of liposome-mediated delivery and expression of foreign DNA in cultured chick embryo, HeLa and hepatoma cells has also been demonstrated (Wong *et al.* 1980).

In certain embodiments of the invention, a liposome may be complexed with a hemagglutinating virus (HVJ). This has been shown to facilitate fusion with the cell membrane and promote cell entry of liposome-encapsulated DNA (Kaneda *et al.* 1989). In other embodiments, a liposome may be complexed or employed in conjunction with nuclear non-histone chromosomal proteins (HMG-1) (Kato *et al.* 1991). In yet further embodiments, a liposome may be complexed or employed in conjunction with both HVJ and HMG-1. In other embodiments, a delivery vehicle may comprise a ligand and a liposome.

#### viii) Receptor Mediated Transfection

Still further, a nucleic acid may be delivered to a target cell via receptor-mediated delivery vehicles. These take advantage of the selective uptake of macromolecules by receptor-mediated endocytosis that will be occurring in a target cell. In view of the cell type-specific distribution of various receptors, this delivery method adds another degree of specificity to the present invention.

Certain receptor-mediated gene targeting vehicles comprise a cell receptor-specific ligand and a nucleic acid-binding agent. Others comprise a cell receptor-specific ligand to which the nucleic acid to be delivered has been operatively attached. Several ligands have been used for receptor-mediated gene transfer (Wu and Wu, 1987; Wagner *et al.*, 1990; Perales *et al.*, 1994; Myers, EPO 0273085), which establishes the operability of the technique. Specific delivery in the context of another mammalian cell type has been described (Wu and Wu, 1993; incorporated herein by reference). In certain aspects of the present invention, a ligand will be chosen to correspond to a receptor specifically expressed on the target cell population.

In other embodiments, a nucleic acid delivery vehicle component of a cell-specific nucleic acid targeting vehicle may comprise a specific binding ligand in combination with a liposome. The nucleic acid(s) to be delivered are housed within the liposome and the specific binding ligand is functionally incorporated into the liposome membrane. The liposome will thus specifically bind to the receptor(s) of a target cell and deliver the contents to a cell. Such systems have been shown to be functional using systems in which, for example, epidermal growth factor (EGF) is used in the receptor-mediated delivery of a nucleic acid to cells that exhibit upregulation of the EGF receptor.

In still further embodiments, the nucleic acid delivery vehicle component of a targeted delivery vehicle may be a liposome itself, which will preferably comprise one or more lipids or glycoproteins that direct cell-specific binding. For example, lactosyl-ceramide, a galactose-terminal asialganglioside, have been incorporated into liposomes and observed an increase in the uptake of the insulin gene by hepatocytes (Nicolau *et al.* 1987). It is contemplated that the tissue-specific transforming constructs of the present invention can be specifically delivered into a target cell in a similar manner.

#### ix) Microprojectile Bombardment

Microprojectile bombardment techniques can be used to introduce a nucleic acid into at least one, organelle, cell, tissue or organism (U.S. Patent 5,550,318; U.S. Patent 5,538,880; U.S. Patent 5,610,042; and PCT Application WO 94/09699; each of which is incorporated herein by reference). This method depends on the ability to accelerate DNA-coated microprojectiles to a high velocity allowing them to pierce cell membranes and enter cells without killing them (Klein *et al.*, 1987). There are a wide variety of microprojectile bombardment techniques known in the art, many of which are applicable to the invention.

In microprojectile bombardment, one or more particles may be coated with at least one nucleic acid and delivered into cells by a propelling force. Several devices for accelerating small particles have been developed. One such device relies on a high voltage discharge to generate an electrical current, which in turn provides the motive force (Yang *et al.*, 1990). The microprojectiles used have consisted of biologically inert substances such as tungsten or gold particles or beads. Exemplary particles include those comprised of tungsten, platinum, and preferably, gold. It is contemplated that in some instances DNA precipitation onto metal particles would not be necessary for DNA delivery to a recipient cell using microprojectile bombardment.



However, it is contemplated that particles may contain DNA rather than be coated with DNA. DNA-coated particles may increase the level of DNA delivery via particle bombardment but are not, in and of themselves, necessary.

For the bombardment, cells in suspension are concentrated on filters or solid culture medium. Alternatively, immature embryos or other target cells may be arranged on solid culture medium. The cells to be bombarded are positioned at an appropriate distance below the macroprojectile stopping plate.

An illustrative embodiment of a method for delivering DNA into a cell (e.g., a plant cell) by acceleration is the Biolistics Particle Delivery System, which can be used to propel particles coated with DNA or cells through a screen, such as a stainless steel or Nytex screen, onto a filter surface covered with cells, such as for example, a monocot plant cells cultured in suspension. The screen disperses the particles so that they are not delivered to the recipient cells in large aggregates. It is believed that a screen intervening between the projectile apparatus and the cells to be bombarded reduces the size of projectiles aggregate and may contribute to a higher frequency of transformation by reducing the damage inflicted on the recipient cells by projectiles that are too large.

## **VI. TREATMENT OF VARIOUS DISEASE STATES**

In accordance with the present invention, applicants propose the use of NKG2D ligands or derivatives thereof to stimulate NKG2D expressing T-cells. In particular, applicants envision the use of such ligands to stimulate immune responses in a variety of clinical situations.

### **A. Obtaining T-Cell Populations**

Antigen-specific T-cells can be directly isolated from peripheral blood or tissue from patients using, for example, HLA-peptide complex tetramer technology (Altman *et al.*, 1996) and *in vitro* expanded using established culture conditions in the presence of irradiated antigen-presenting cells, solid-phase anti-NKG2D and cytokines. Additional methods may include FACS sorting and/or techniques based on magnetic beads coupled with antibodies to enrich desired T-cell populations (Groh *et al.* 1998). Large numbers of such T-cell populations with demonstrated antigen-specificity can subsequently be infused into patients. Another disease treatment platform is envisioned by using derivatives of anti-NKG2D antibody, such as bi-specific antibodies, or of suitably engineered ligands, to directly target T-cells systemically or locally in the body, with

the goal to enhance their ability to execute effector functions (cytotoxicity and cytokine release) and to induce limited proliferation.

#### **B. Treatment of Cancer**

5 In accordance with one embodiment of the present invention, there is provided a method for treating various cancers, including breast cancer, lung cancer, prostate cancer, cervical cancer, testicular cancer, brain cancer, renal cancer, liver cancer, stomach cancer, colon cancer, pancreatic cancer, head & neck cancer, skin cancer and ovarian cancer. As discussed above, appropriate cell populations are stimulated using NKG2D ligands as described elsewhere in this  
10 document. Such populations may be stimulated *in vivo* by administration of ligands as part of a suitable pharmaceutical preparation. Alternatively, an appropriate cell population may be isolated from the cancer patient, stimulated *ex vivo*, and then reinfused into the patient. The infusion of stimulated cells may be intratumoral, into the tumoral vasculature, regional to the tumor, or systemically via intravenous or intraarterial infusion. Systemic administration is particularly  
15 advantageous when attempting to prevent or treat metastatic tumors.

#### **C. Treatment of Viral Infection**

In another embodiment, the present invention provides for treatment or prevention of viral infection. Viruses contemplated as treatable using methods of the present invention include  
20 cytomegalovirus, herpesvirus, human immunodeficiency virus, influenza virus and any others. Treatment is envisioned as described above, by infusion of *ex vivo* expanded T-cells derived from a patient or by *in vivo* targetting of specific T-cells using suitable derivatives of anti-NKG2D antibody or ligands. This method may be of particular use with patients who are partially immunocompromised as a result of therapeutic treatment (radiation, chemotherapy,  
25 cytostatica) or disease (AIDS), by providing mobilization of compromised T-cell function.

#### **D. Stimulation of Cytokine Production**

In yet another embodiment, the present invention provides for methods of stimulating the secretion of cytokines by lymphocytes. These cytokines include interferon- $\gamma$  (IFN- $\gamma$ ), tumor  
30 necrosis factor- $\alpha$  (TNF- $\alpha$ ), IL-2, IL-4 and GM-CSF, among others (Groh *et al.* 1998, 1999; see FIGS. 5-7). The stimulation of lymphokine production by anti-NKG2D antibody or a ligand

derivative facilitates the proliferation of specific T-cell populations *in vitro* and may enhance their effector functions *in vivo*.

## **VII. SCREENING FOR LIGANDS OF NKG2D**

5        Within certain embodiments of the invention, methods are provided for screening for compounds that bind to, and hence activate, NKG2D. Within one example, a screening assay is performed in which cells expressing NKG2D are exposed to a test substance under suitable conditions and for a time sufficient to permit activation thereof. Activation may be measured, for example, by cellular proliferation, cytokine expression, or target cell lysis. Generally, the test  
10        substance is added in the form of a purified agent.

      An alternative embodiment is a binding assay. Using an NKG2D receptor, one may measure binding to the receptor via a variety of methods, including alteration in electrophoretic mobility of the NKG2D (or fragment), competitive binding for NKG2D (as measured by loss of signal for labeled competitor), or any other suitable method. Also, industrial scale screenings of  
15        commercially available drug banks and peptide libraries for compounds binding to NKG2D are envisioned.

## **VIII. KIT COMPONENTS**

      All the essential materials and reagents required for stimulating NKG2D, or fusion  
20        molecules thereof, may be assembled together in a kit. Such kits generally will comprise, in suitable means, distinct containers for each individual ligand. Such kits also may comprise, in suitable distinct containers, buffer for dilution of ligand. Other reagents may be growth factors or lymphokines/cytokines for culturing of stimulated cells.

## **IX. PHARMACEUTICAL COMPOSITIONS**

25        For use according to the present application, it may be necessary to prepare pharmaceutical compositions – NKG2D ligands - in a form appropriate for the intended application. Generally, this will entail preparing compositions that are essentially free of pyrogens, as well as other impurities that could be harmful to cells of humans or animals.

30        One will generally desire to employ appropriate salts and buffers suitable for dilution of ligands. Buffers also will be employed when recombinant cells are introduced into a patient. Aqueous compositions of the present invention comprise an effective amount of the vector to

cells, dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. Such compositions also are referred to as inocula. The phrase "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce adverse, allergic, or other untoward reactions when administered to an animal or a human. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutically-active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the vectors or cells of the present invention, its use in therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

The expression vectors and delivery vehicles of the present invention may include classic pharmaceutical preparations. Administration of these compositions according to the present invention will be via any common route so long as the target tissue is available via that route. This includes oral, nasal, buccal, rectal, vaginal or topical. Alternatively, administration may be by intratumoral, intradermal, subcutaneous, intramuscular, intraperitoneal or intravenous injection. Such compositions would normally be administered as pharmaceutically acceptable compositions, described *supra*.

The active compounds may also be administered parenterally or intraperitoneally. Solutions of the active compounds as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of

dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various anti-bacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

For oral administration the polypeptides of the present invention may be incorporated with excipients and used in the form of non-ingestible mouthwashes and dentifrices. A mouthwash may be prepared incorporating the active ingredient in the required amount in an appropriate solvent, such as a sodium borate solution (Dobell's Solution). Alternatively, the active ingredient may be incorporated into an antiseptic wash containing sodium borate, glycerin and potassium bicarbonate. The active ingredient may also be dispersed in dentifrices, including: gels, pastes, powders and slurries. The active ingredient may be added in a therapeutically effective amount to a paste dentifrice that may include water, binders, abrasives, flavoring agents, foaming agents, and humectants.

The compositions of the present invention may be formulated in a neutral or salt form. Pharmaceutically-acceptable salts include the acid addition salts (formed with the free amino

groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as injectable solutions, drug release capsules and the like.

For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

## **X. EXAMPLES**

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

## EXAMPLE 1: METHODS

### CMV infection of fibroblasts and endothelial cells, antibodies and flow cytometry.

Primary human fibroblast (HF) cultures were established from skin biopsies of healthy individuals and grown in Waymouths media (Gibco) supplemented with 10% fetal bovine serum (FBS; Hyclone) and standard concentrations of penicillin, streptomycin and glutamine. Human umbilical vein endothelial cells (HUVEC) were grown on fibronectin-coated plates (Upstate Technologies) in RPMI (Gibco), 20% FBS, HEPES (10 mM), non-essential amino acids (0.1 mM; Gibco), endothelial cell growth supplement (50 µg/ml; Becton Dickinson), sodium pyruvate (1 mM), glutamine (2 mM), and antibiotics (penicillin, streptomycin and fungizone). Early (1-5 passage) cells grown to confluency were infected with CMV strain AD169 [5 plaque-forming units (pfu)/cell; American Type Culture Collection (ATCC)] or strain VHL/e (2 pfu/cell) (Waldmann *et al.*, 1989). Control infections were with UV-irradiated ( $10^6$  joules/100 µl virus stock) AD169, which produced positive immunostaining for CMV pp65 (mAb anti-CMV pp65; Virostat) but no staining for IE-1 (mAb NEA-9221; NEN Life Science Products), and with heat-inactivated AD169 and mock-infected cell lysate stock. HF and HUVEC were stained before and at various time points after infection or control or mock infection with mAb 6D4 (anti-MICA and MICB; Groh *et al.* 1998), mAb W6/32 (anti-pan MHC class I; Parham *et al.*, 1979), or Ig isotype-matched control antibody (IgG2a) and examined by indirect immunofluorescence using phycoerythrin (PE)-conjugated goat F(ab')<sub>2</sub> anti-mouse Ig (Biosource) and flow cytometry.

**Immunohistochemistry of CMV-infected cell cultures and lung tissue.** Cytospin preparations of infected and control HF were fixed in cold acetone, blocked with 20% normal goat and 20% human serum in Tris-buffered saline, and incubated with mAb NEA-9221 (anti-CMV IE-1), anti CMV pp65 mAb, or isotype control Ig. Bound antibody was stained with biotin-goat anti-mouse F(ab')<sub>2</sub> Ig (Jackson ImmunoResearch Laboratories) and Streptavidin Alexa<sup>TM</sup> 594 conjugate (Molecular Probes). Infected and control HUVEC monolayers grown on glass chamber well slides (Nalge Nunc International Corp) were acetone-fixed, stained for MIC expression with mAb 6D4 as described above, blocked with Avidin/Biotin Blocking Kit (Vector Laboratories), and double-stained with mAb NEA-9221, biotin-goat anti-mouse F(ab')<sub>2</sub> Ig and Streptavidin CY<sup>TM</sup> conjugate (Jackson ImmunoResearch Laboratories). Nuclei were stained with 4'-6 diamino-2-phenylindole (5 µg/ml; Sigma). Samples were examined using a Delta Vision

system (Applied Precision). Cryostat sections of OCT compound-embedded and snap-frozen CMV interstitial pneumonia autopsy specimens, post-transplant for treatment of chronic myeloid leukemia (CML), were air-dried and acetone-fixed, and stained with mAb 6D4 and mAb CCH2 (anti-CMV delayed early DNA-binding protein p52; Dako) using the Envision Doublestain System (Dako) with the diamino benzidine and Fast Red peroxidase substrates as described by the manufacturer.

**Generation and maintenance of the CMV pp65-specific T-cell clones and isolation of peripheral blood CD8<sup>+</sup>  $\alpha\beta$  T-cells.** The CD8  $\alpha\beta$  T-cell clones (HLA-A2-restricted clones 88C7-470, 94C10-12, 19D1-66, 4H6-254, 59C11-292 and 2E9-269; HLA-A1-restricted clones 8E8-403, 21D9-306 and 30F4-297) were generated from short-term CMV-specific cytotoxic T-cell lines as previously described (McLaughlin-Taylor *et al.*, 1994; Gilbert *et al.*, 1996). In brief, peripheral blood mononuclear cells (PBMC) from CMV seropositive volunteers were stimulated with autologous fibroblasts infected with AD169 (at a multiplicity of infection of 5) at a ratio of 1:20 in RPMI media supplemented with 10% human serum, 2-mercaptoethanol (25  $\mu$ M), glutamine, penicillin and streptomycin. Cultures were restimulated after 7 days with autologous CMV-infected fibroblasts, in the presence of autologous  $\gamma$ -irradiated PBMC and recombinant IL-2 (Proleukin-2, 5U/ml; Chiron). After 7 additional days, CD4<sup>+</sup> T-cells were depleted using CD4 Dynabeads (Dyna) and enriched CD8<sup>+</sup> T-cells plated (0.5 cells per well) and grown as described above. CD8  $\alpha\beta$  T-cell clones were tested for anti-CMV specificity in chromium release assays and further expanded in the presence of  $\gamma$ -irradiated PBMC, anti-CD3 (OKT3, 30 ng/ml; Orthobiotec) and IL-2 (50 U/ml). McLaughlin-Taylor *et al.* (1994); Gilbert *et al.* (1996).

CD28/CD8 T-cells were isolated from unseparated peripheral blood from healthy donors by negative selection using the CD8 T-cell enrichment cocktail RosetteSep<sup>TM</sup> (StemCell Technologies) and by depletion of CD28 T-cells using magnetic Pan Mouse IgG Dynabeads (Dyna) precoated with anti-CD28 (mAb 9.3; Hara *et al.*, 1985) on a magnetic particle concentrator (Dyna). By flow cytometry, the CD28/CD8 T-cells were of at least 98% purity.

**Cytotoxicity, cytokine release and T-cell proliferation assays.** T-cell cytolytic activity was tested in standard 4-h <sup>51</sup>Cr-release assays with labeled targets cells that included HF (typed for HLA-A1 or -A2) that were infected with CMV AD169 or mock-infected, and transfectants of the B-lymphoblastoid C1R cell line expressing HLA-1 or -A2 alone or together with MICA (Groh *et al.*, 1998). Before exposure to the HLA-A1- or -A2-restricted CMV pp65-specific T-cell clones, the transfectants were pulsed with the specific naturally processed pp65 9-mer peptides



YSEHPTFTS and NLVPMVATV, respectively (Wills *et al.*, 1996), at the concentrations indicated in the figure legends. For blocking experiments, effector or target cells were incubated with saturating amounts of mAb 1D11 (anti-NKG2D), W6/32 (anti-pan HLA) or mAb 6D4 (anti-MIC), either alone or in combination, or with control Ig, for 30 min before exposure to T-cells. Assays were performed in triplicate and results scored in percent specific lysis according to the standard formula.

In the cytokine release assays, T-cells ( $10^5$  cells per well) were stimulated with equal numbers of C1R-HLA-2 or C1R-HLA-2-MICA transfectants pulsed with the pp65 peptide at the indicated concentrations, in the presence or absence of mAb 6D4, mAb 1D11, or control Ig. In the mAb triggering experiments, the T-cells were stimulated with solid-phase anti-CD3 (OKT3; Orthobiotec) with or without mAb 1D11 or control Ig. Antibodies were plate-bound by precoating 96-well flat bottom microtiter plates with goat anti-mouse Fc-specific F(ab')<sub>2</sub> Ig (Jackson Immunoresearch Laboratories). T-cell supernatants from triplicate wells were harvested and pooled after 24 and 48 h of culture, and the amounts of secreted IFN- $\gamma$ , TNF- $\alpha$ , GM-CSF, IL-2 and IL-4 were determined by commercial ELISA with matched antibody in relation to cytokine standard pairs (R & D Systems).

T-cell proliferation was measured with rested T-cell clones ( $10^5$  cells per well; 14-21 days after stimulation) or with freshly isolated peripheral blood CD8/CD28<sup>-</sup>  $\alpha\beta$  T-cells after activation with plate-bound mAbs as described above. Cultures were pulsed with [<sup>3</sup>H]thymidine on day 3 and harvested 16 h later using a Micromate cell harvester (Packard). Incorporated radioactivity was determined using Unifilter GF/C plates and a Topcount (Packard).

**HLA-A2 tetramer and intracellular cytokine staining of CMV pp65-specific T-cells from peripheral blood.** The HLA-A2-peptide complex tetramers were produced similar to the original method (Altman *et al.*, 1996); Callan *et al.*, 1998). In brief, the extracellular domains of HLA-A2 with a carboxyterminal BirA enzyme substrate site and  $\beta_2$ -microglobulin ( $\beta_2m$ ) were expressed in bacteria and purified from inclusion bodies. Complexes of HLA-A2,  $\beta_2m$  and pp65 peptide NLVPMVATV were refolded *in vitro* in the presence of protease inhibitors, biotinylated and HPLC-purified. Tetramers were obtained by treatment with streptavidin-PE at a molar ratio of 4:1. CD8  $\alpha\beta$  T-cells were isolated from peripheral blood of a healthy donor previously typed for HLA-A2 and screened for high numbers of pp65-specific T-cells, using negative selection with RosetteSep<sup>TM</sup> (StemCell Technologies). T-cells ( $2 \times 10^6$ ; >98% CD8  $\alpha\beta$  T-cells) were stimulated with equal numbers of C1R-HLA-A2 or C1R-HLA-A2-MICA cells pulsed with the

pp65 peptide (500 nM) in the presence of Monensin (0.6  $\mu$ l/ml; Golgistop, Pharmingen) in 96-well round bottom plates ( $0.2 \times 10^6$  cells/well) for 8 h at 37°C. Thereafter, pp65-specific-T-cells were identified by staining with the PE-conjugated tetramer reagent, stained with anti-CD28-FITC (Immunotech), fixed and permeabilized using a Cytotfix/Cytoperm Plus Kit (Pharmingen), and stained for intracellular IL-2 with an allophycocyanin (APC)-conjugated mAb (Pharmingen). Cells were analyzed with a Becton-Dickinson FACS Vantage cytometer.

## EXAMPLE 2: RESULTS

**Induction of MIC expression by CMV infection.** Surface expression of MIC was monitored on human fibroblasts infected at high multiplicity with the CMV strain AD169 using the monoclonal antibody (mAb) 6D4, which is specific for MICA and MICB, and flow cytometry (Groh *et al.* 1998). From 24 to 72 h after infection, surface MIC increased progressively to amounts that were about 10-fold higher than those on mock-infected control cells. Concurrently, expression of MHC class I decreased by a similar factor (FIG. 1A). Productive infection of all fibroblasts was confirmed by staining for the CMV immediate-early nuclear antigen-1 (IE-1); moreover, expression of MIC was not induced by UV-inactivated virus, which can enter cells but cannot productively infect (data not shown). Similar results were obtained with endothelial cells, which was physiologically significant since endothelium is a well established site of CMV infection in a chronically infected host. Contour profiles of endothelial cell cultures that were incompletely infected with the viral strain VHL/e at low multiplicity displayed two cell populations with inversely correlated expression levels of MIC and MHC class I (FIG. 1B). Two-color immunostainings of the partially infected endothelial cell monolayers demonstrated that induction of MIC was strictly associated with expression of viral IE-1 (FIG. 2A). These results show that productive infection by different CMV strains potently increases the expression of MIC, presumably as a consequence of the cell stress response. Induction of MIC by CMV was confirmed *in vivo*, by two-color immunohistochemistry stainings of lung sections from patients with CMV interstitial pneumonia. All of three samples examined included multiple foci of cytomegalic cells that exhibited intense staining for both the CMV delayed-early DNA-binding protein p52 and MIC (FIG. 2B). This observation extended the results obtained in cell culture and supported the physiological significance of the virus-induced expression of MIC.

**NKG2D-MIC interaction augments cytolytic responses.** Although CMV gene products severely impair MHC class I antigen processing and expression, the virus is under immunological control as reflected by the frequent reactivation of CMV and progression to fatal disease in immunocompromised patients (Riddell *et al.*, 1992; Riddell, 1995). Hence, the inventors investigated whether the induced expression of MIC could compensate for deficient MHC class I function, by positively modulating viral antigen-specific CD8  $\alpha\beta$  T-cell responses *via* engagement of NKG2D. This notion was based on the ability of NKG2D to function as an activating receptor in antibody-dependent cytotoxicity assays, although its contribution, if any, to TCR-dependent T-cell activation is unknown (Bauer *et al.*, 1999). A total of nine CD8  $\alpha\beta$  T-cell clones (all CD28<sup>+</sup>, CD94<sup>+</sup>, NKG2D<sup>+</sup>; KIR2DL1<sup>-</sup>, KIR2DL2<sup>-</sup>, KIR2DL3<sup>-</sup>; KIR2S1<sup>-</sup>, KIR2S2<sup>-</sup>; KIR3DL1<sup>-</sup>, KIR3DL2<sup>-</sup>), which recognize defined epitopes of the CMV pp65 matrix protein in the context of HLA-A1 or -A2 (McLaughlin-Taylor, 1994; Gilbert *et al.*, 1996), were tested in cytotoxicity assays using autologous or HLA-matched fibroblasts infected with CMV AD169 as targets. At 12 h post-infection, a time point at which the surface levels of MHC class I and MIC were yet unchanged (FIG. 3A & 3B), T-cell cytotoxicity was maximal and could be inhibited by mAb against MHC class I (mAb W6/32; pan anti-HLA-A, -B and -C; Parham *et al.* 1979) but not by mAbs specific for MIC (mAb 6D4; Groh *et al.*, 1998) or NKG2D (mAb 1D11; Bauer *et al.*, 1999) (FIG. 3C & 3F). Thus, under the conditions of undiminished MHC class I and low MIC expression, NKG2D was not involved in cytolytic T-cell function. By contrast, at 72 h post-infection, when MHC class I expression was impaired and MIC reached maximum surface levels (FIG. 3A & 3B), mAb masking of MIC or NKG2D substantially reduced target cell lysis (FIG. 3C & 3F). This was not due to TCR-independent activation resulting from the increased expression of MIC and triggering of NKG2D since no cytotoxicity was observed when T-cell clones were tested against HLA-mismatched virus-infected fibroblasts (FIG. 3D & 3E). Moreover, mAb masking of MHC class I, MIC and NKG2D altogether was additive in lysis inhibition (FIG. 3C & 3F). Hence, these results suggested that engagement of NKG2D augmented CMV-specific cytotoxic T-cell responses under conditions of suboptimal MHC-antigen stimulation of TCR. This was confirmed using C1R cell transfectants expressing HLA-A2 alone or together with MICA, which were pulsed with titrated concentrations of the CMV pp65 peptide and tested against five of the antigen-specific T-cell clones. At optimal peptide concentrations, both target cell lines were lysed equally well and mAb against MICA or NKG2D had no inhibitory effects (FIG. 4). However, with increasingly limiting peptide concentrations,

the responses against C1R-A2-MICA cells remained substantially stronger than those against the targets lacking MICA, which declined rapidly. This functional augmentation was abrogated by mAbs against MICA or NKG2D and was qualitatively similar to the differences observed with the CMV-infected fibroblasts late versus early after infection. Altogether, these results indicated that NKG2D could enhance anti-CMV and presumably other cytotoxic CD8  $\alpha\beta$  T-cell responses.

**T-cell costimulation by NKG2D.** The inventors' observations, together with previous data indicating that NKG2D may signal *via* its adaptor protein DAP10 in a similar pathway as CD28, raised the question of whether NKG2D could costimulate T-cell activation, by induction of cytokine production and T-cell proliferation. Peptide-pulsed C1R-A2-MICA cells were substantially more potent stimulators (100-500%) of interferon- $\gamma$  (IFN- $\gamma$ ), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), IL-4, and granulocyte/macrophage-colony stimulating factor (GM-CSF) release by the A2-restricted pp65-specific T-cell clones than identically treated C1R-A2 cells lacking MICA (FIG. 5A, 5B & 5D, and data not shown). These results were highly reproducible in three independent experiments and were representative of five different T-cell clones tested. Distinct from the results obtained with the cytotoxicity assays, the cytokine responses were superinduced even when MHC-antigen stimulation of TCR by C1R-A2 cells pulsed with saturating peptide concentrations (10-100 nM) was optimal. CD28/CD8  $\alpha\beta$  T-cells, the phenotype common to all of the T-cells used in this study so far, fail to produce IL-2 in response to triggering of TCR-CD3 (Azuma *et al.* 1993). Hence, it was of particular interest that expression of MICA on the stimulator cells resulted in induction of IL-2, which was not detectably produced by T-cells exposed to the MICA-negative cells (FIG. 5C). In all of these experiments, mAb masking of MICA abrogated the augmentation or *de novo* induction of cytokine production. By contrast, in the presence of anti-NKG2D mAb, the amounts of cytokines were either variably increased or unchanged (data not shown). Thus, in these long-term (24-48 h) cytokine release assays, the anti-NKG2D mAb had at least weak stimulatory capacity, either *via* binding to NKG2D in solution or after becoming crosslinked, or both. This was opposite to the inhibitory effect of the same soluble mAb in the short-term (4 h) cytotoxicity assays, presumably because the previously observed high affinity interactions of MIC with NKG2D were critical in enhancing effector-target cell contacts and in triggering cytotoxicity (Bauer *et al.* 1999). The cytokine release observations made with the five T-cell clones could be replicated with CMV-specific CD28/CD8  $\alpha\beta$  T-cells identified by staining with HLA-A2-peptide pp65 tetramers among freshly isolated peripheral blood CD8<sup>+</sup> T-cells (FIG. 6A). After short-term antigen stimulation in the

presence but not in the absence of MIC, a proportion of these T-cells showed positive staining for intracellular IL-2 (FIG. 6B & 6C). Collectively, these results clearly supported a costimulatory function of NKG2D.

Further evidence for costimulation of CD28/CD8  $\alpha\beta$  T-cells by NKG2D was obtained using titrated concentrations of solid-phase anti-CD3 with or without anti-NKG2D mAb to stimulate cytokine secretion and proliferation by the pp65-specific T-cells. All of four T-cell clones tested produced no or little IL-2 and IL-4 and showed modest dose-dependent proliferative responses upon triggering with anti-CD3 mAb alone. In the additional presence of anti-NKG2D, however, IL-2 and IL-4 were potently induced and T-cell proliferation was about four-fold amplified (FIG. 7 A & 7B, and data not shown). No effect was seen when anti-NKG2D was used in the absence of anti-CD3. A similar synergistic induction of proliferation was recorded with freshly isolated peripheral blood CD28/CD8<sup>+</sup> T-cells (FIG. 7C). Thus, NKG2D was a potent costimulator of TCR-CD3 complex-dependent T-cell activation capable of substituting for CD28.

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All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

## REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

5

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